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(54) Title: ADENOVIRUS VECTORS CONTAINING HETEROLOGOUS TRANSCRIPTION REGULATORY ELEMENTS AND METHODS OF USING SAME (57) Abstract Replication-competent adenovirus vectors specific for target cells and methods of use of such viruses are provided. These adenoviruses comprise a first adenoviral gene under control of a cell specific heterologous (i.e., non-adenoviral) transcriptional regulatory element (TRE) and at least a second gene under control of a second heterologous TRE, where the heterologous TREs are different from each other in polynucleotide sequence but functional in the same cell. The adenoviral gene can be, for example, a gene required for adenoviral replication. The second gene can be, for example, a second adenoviral gene or a transgene, such as a gene which can contribute to cytotoxicity in the target cell. Adenoviral replication can be restricted to target cells in which the heterologous TREs are functional and thus, the adenovirus vectors can provide selective cytotoxicity to the target cells, particularly neoplastic cells.		

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**ADENOVIRUS VECTORS CONTAINING HETEROLOGOUS TRANSCRIPTION
REGULATORY ELEMENTS AND METHODS OF USING SAME**

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Serial No. 60/039,762, filed March 3, 1997, U.S. Serial No. 60/039,763, filed March 3, 1997 and U.S. Serial No. 60/054,523, filed August 8, 1997.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER
10 FEDERALLY SPONSORED RESEARCH

(Not applicable)

TECHNICAL FIELD

15 This invention relates to cell transfection using adenovirus vectors, providing replication-competent adenovirus vectors and methods of their use. More specifically, it relates to cell-specific replication of adenovirus vectors in cells through the use of cell-specific, non-adenoviral transcriptional regulatory elements.

BACKGROUND ART

20 In spite of numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Neoplasia resulting in benign tumors can usually be completely cured by
25 removing the mass surgically. If a tumor becomes malignant, as manifested by invasion of surrounding tissue, it becomes much more difficult to eradicate. Once a malignant tumor metastasizes, it is much less likely to be eradicated.

30 Excluding basal cell carcinoma, there are over one million new cases of cancer per year in the United States alone, and cancer accounts for over one half million deaths per year in this country. In the world as a whole, the five most common cancers are those of lung, stomach, breast, colon/rectum, and uterine cervix, and the total number of new cases per year is over 6 million.

Lung cancer is one of the most refractory of solid tumors because inoperable cases are up to 60% and the 5-year survival is only 13%. In particular, adenocarcinomas, which comprise about one-half of the total lung cancer cases, are mostly chemo-radioresistant. Gastric (i.e., stomach) carcinoma is one of the most prevalent forms of cancers in East Asia, including Japan and Korea. Although extensive surgical operations have been combined with chemotherapy and immunotherapy, the mortality of gastric cancer is still high, due to carcinomatous peritonitis and liver metastasis at advanced stages. Colorectal cancer is the third most common cancer and the second leading cause of cancer deaths. Pancreatic cancer is virtually always fatal. Thus, current treatment prospects for many patients with these carcinomas are unsatisfactory, and the prognosis is poor.

Hepatocellular carcinoma (HCC or malignant hepatoma) is one of the most common cancers in the world, and is especially problematic in Asia. Treatment prospects for patients with hepatocellular carcinoma are dim. Even with improvements in therapy and availability of liver transplant, only a minority of patients are cured by removal of the tumor either by resection or transplantation. For the majority of patients, the current treatments remain unsatisfactory, and the prognosis is poor.

Breast cancer is one of the most common cancers in the United States, with an annual incidence of about 182,000 new cases and nearly 50,000 deaths. In the industrial nations, approximately one in eight women can expect to develop breast cancer. The mortality rate for breast cancer has remained unchanged since 1930. It has increased an average of 0.2% per year, but decreased in women under 65 years of age by an average of 0.3% per year. See e.g., Marchant (1994) *Contemporary Management of Breast Disease II: Breast Cancer*, in: *Obstetrics and Gynecology Clinics of North America* 21:555-560; and Colditz (1993) *Cancer Suppl.* 71:1480-1489.

Despite ongoing improvement in the understanding of the disease, breast cancer has remained resistant to medical intervention. Most clinical initiatives are focused on early diagnosis, followed by conventional forms of intervention, particularly surgery and chemotherapy. Such interventions are of limited success, particularly in patients where the tumor has undergone metastasis. There is a pressing need to improve the arsenal of therapies available to provide more precise and more effective treatment in a less invasive way.

Prostate cancer is the fastest growing neoplasm in men with an estimated 244,000 new cases in the United States being diagnosed in 1995, of which approximately 44,000 deaths will result. Prostate cancer is now the most frequently diagnosed cancer in men.

Prostate cancer is latent; many men carry prostate cancer cells without overt signs of disease. It is associated with a high morbidity. Cancer metastasis to bone (late stage) is common and is almost always fatal.

Current treatments include radical prostatectomy, radiation therapy, hormonal ablation and chemotherapy. Unfortunately, in approximately 80% of cases, diagnosis of prostate cancer is established when the disease has already metastasized to the bones, thus limiting the effectiveness of surgical treatments. Hormonal therapy frequently fails with time with the development of hormone-resistant tumor cells. Although chemotherapeutic agents have been used in the treatment of prostate cancer, no single agent has demonstrated superiority over its counterparts, and no drug combination seems particularly effective. The generally drug-resistant, slow-growing nature of most prostate cancers makes them particularly unresponsive to standard chemotherapy.

A major, indeed the overwhelming, obstacle to cancer therapy is the problem of selectivity; that is, the ability to inhibit the multiplication of tumor cells, while leaving unaffected the function of normal cells. For example, in prostate cancer therapy, the therapeutic ratio, or ratio of tumor cell killing to normal cell killing of traditional tumor chemotherapy, is only 1.5:1. Thus, more effective treatment methods and pharmaceutical compositions for therapy and prophylaxis of neoplasia are needed.

Of particular interest is development of more specific, targeted forms of cancer therapy, especially for cancers that are difficult to treat successfully. In contrast to conventional cancer therapies, which result in relatively non-specific and often serious toxicity, more specific treatment modalities attempt to inhibit or kill malignant cells selectively while leaving healthy cells intact.

One possible treatment approach for many of these cancers is gene therapy, whereby a gene of interest is introduced into the malignant cell. See, for gene therapy for prostate cancer, Boulikas (1997) *Anticancer Res.* 17:1471-1505. The gene of interest may encode a protein which converts into a toxic substance upon treatment with another compound, or an enzyme that converts a prodrug to a drug. For example, introduction of the herpes simplex gene encoding thymidine kinase (HSV-tk) renders cells conditionally sensitive to ganciclovir. Zijlstra et al. (1989) *Nature* 342: 435; Mansour et al. (1988) *Nature* 336: 348; Johnson et al. (1989) *Science* 245: 1234; Adair et al. (1989) *Proc. Natl. Acad. Sci. USA* 86: 4574; Capecchi (1989) *Science* 244: 1288. Alternatively, the gene of interest may encode a compound that is directly toxic, such as diphtheria toxin. For these

treatments to be rendered specific to cancer cells, the gene of interest can be under control of a transcriptional regulatory element (TRE) that is specifically (i.e., preferentially) activated in the cancer cells. Cell or tissue specific expression can be achieved by using a TRE with cell-specific enhancers and/or promoters. See generally Huber et al. (1995) *Adv. Drug Delivery Reviews* 17:279-292.

A variety of viral and non-viral (e.g., liposomes) vehicles, or vectors, have been developed to transfer these genes. Of the viruses proposed for gene transfer, adenoviruses are among the most easily produced and purified. Adenovirus also has the advantage of effecting high efficiency of transduction and does not require cell proliferation for efficient transduction of cell. In addition, adenovirus can infect a wide variety of cells *in vitro* and *in vivo*. For general background references regarding adenovirus and development of adenoviral vector systems, see Graham et al. (1973) *Virology* 52:456-467; Takiff et al. (1981) *Lancet* 11:832-834; Berkner et al. (1983) *Nucleic Acid Research* 11: 6003-6020; Graham (1984) *EMBO J* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; and Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

When used as gene transfer vehicles, adenovirus vectors are often designed to be replication-defective and are thus deliberately engineered to fail to replicate in the target cells of interest. In these vehicles, the early adenovirus gene products E1A and/or E1B are deleted and provided *in trans* by the packaging cell line 293. Graham et al. (1987) *J. Gen. Virol* 36:59-72; Graham (1977) *J. Genetic Virology* 68:937-940. The gene to be transduced is commonly inserted into adenovirus in the E1A and E1B region of the virus genome. Bett et al. (1994). Replication-defective adenovirus vectors as vehicles for efficient transduction of genes have been described by, inter alia, Stratford-Perricaudet (1990) *Human Gene Therapy* 1:241-256; Rosenfeld (1991) *Science* 252:431-434; Wang et al. (1991) *Adv. Exp. Med. Biol.* 309:61-66; Jaffe et al. (1992) *Nat. Gen.* 1:372-378; Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Rosenfeld et al. (1992) *Cell* 68:143-155; Stratford-Perricaudet et al. (1992) *J. Clin. Invest.* 90:626-630; Le Gal Le Salle et al. (1993) *Science* 259:988-990; Mastrangeli et al. (1993) *J. Clin. Invest.* 91:225-234; Ragot et al. (1993) *Nature* 361:647-650; Hayaski et al. (1994) *J. Biol. Chem.* 269:23872-23875; Bett et al. (1994).

The virtually exclusive focus in development of adenoviral vectors for gene therapy is use of adenovirus merely as a vehicle for introducing the gene of interest, not as an effector in itself. Replication of adenovirus has been viewed as an undesirable result,

largely due to the host immune response. In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses at two levels. First, the capsid proteins of the adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently expressed in transduced cells, eliciting cellular immunity. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes, suicide genes, or genes which convert prodrug to an active drug has been limited by the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle as well as the transient nature of gene expression.

Adenoviruses generally undergo an effective lytic replication cycle following infection of a host cell. In addition to lysing the infected cell, the replicative process of adenovirus blocks the transport and translation host cell mRNA thus inhibiting protein synthesis of the infected cell. For a review of adenoviruses and adenovirus replication, see Shenk, T. and Horwitz, M.S., *Virology*, third edition, Fields, B.N. et al., eds., Raven Press Limited, New York (1996), Chapters 67 and 68, respectively.

Taking advantage of the cytotoxic effects associated with adenovirus replication, replication-competent adenovirus vectors have recently been described as agents for effecting selective cell growth inhibition. See Henderson et al., U.S. Patent No. 5,698,443; Hallenbeck et al., WO 96/17053. In such systems, a cell-specific transcriptional regulatory element (TRE) controls the expression of a gene essential for viral replication, and thus, viral replication is limited to a cell population in which the TRE is functional. For example, an attenuated, replication-competent adenovirus (CN706) has been generated by inserting the prostate-specific antigen (PSA) promoter and enhancer (PSE-TRE) upstream of the E1A transcription unit in adenovirus serotype 5 (Ad5). CN706 demonstrates selective cytotoxicity toward PSA expressing cells *in vitro* and *in vivo*. Rodriguez et al. (1997). *Cancer Res.* 57:2559-2563.

In sum, there is a need for vector constructs that are capable of eliminating essentially all cancerous cells in a minimum number of administrations before specific immunological response against the vector prevents further treatment. Particularly, there is a continuing serious need for improved replication-competent adenovirus vectors in which cell-specific replication can be further elevated, while minimizing the extent of replication in non-target (i.e., non-cancerous cells). The present invention provides selectively replicating adenovirus vectors that can be employed in these contexts.

SUMMARY OF THE INVENTION

The present invention provides an adenovirus vector comprising a first adenovirus gene under transcriptional control of a first heterologous transcriptional regulatory element (TRE) and at least a second gene under transcriptional control of a second heterologous TRE, wherein the first heterologous TREs is cell-specific, the first heterologous TRE is different from the second heterologous TRE and the heterologous TREs are functional in the same cell.

In one aspect, the invention provides an adenovirus vector in which the cell specific heterologous TRE controls the transcription of a gene essential for adenovirus replication.

In another aspect, the invention provides an adenovirus vector in which the second heterologous TRE controls the transcription of a transgene.

In some embodiments, the invention provides adenovirus vector(s) complexed with a hydrophilic polymer ("masking agent") to create a masked adenovirus. The hydrophilic polymer is attached (covalently or non-covalently) to the capsid proteins of the adenovirus, particularly the hexon and fiber proteins. In preferred embodiments, the adenovirus vectors of the instant invention are complexed with masking agents to create masked adenovirus vectors. In further preferred embodiments, the masking agent is polyethyleneglycol (PEG) covalently linked to an adenovirus vector of the instant invention.

The invention further provides host cells containing the adenovirus vectors of the invention.

Further provided are methods of using the adenoviral vectors of the invention. In one aspect, methods are provided for using the adenovirus vectors described herein which entail introducing these vector(s) into a cell.

In another aspect, methods are provided for conferring selective cytotoxicity on a cell which allows the heterologous TREs to function that entail contacting the cells with an adenovirus vector described herein, wherein the adenovirus vector enters the cell.

In another aspect, methods are provided for suppressing tumor growth, comprising contacting a target cell with an adenovirus vector described herein such that the adenovirus vector enters the cell.

In another aspect, methods are provided for modifying the genotype of a target cell, comprising contacting the cell with an adenovirus vector described herein, wherein the adenovirus vector enters the cell.

In yet another aspect, methods are provided for propagating the adenovirus vectors of the invention, comprising combining the adenovirus vectors with cells which allow the heterologous TREs to function, such that the adenovirus vector enters the cell and is propagated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts schematic diagrams of examples of adenovirus vectors in which various genes are under transcriptional control of various heterologous TREs. The heterologous TREs contained in an adenovirus vector are different from each other and from the endogenous adenovirus TREs. All of the heterologous TREs in an adenovirus vector are functional in the same cell and at least one of the TREs is cell specific.

Figure 2 (A)-(C) are schematic diagrams of examples of adenovirus vectors in which the E1A and E1B genes are under transcriptional control of prostate cell specific heterologous TREs. CN702 is an example of a wild-type Adenovirus type 5 and CN706 contains a single gene (E1A) under control of a single prostate cell specific TRE, one derived from the *PSA* gene.

Figure 3 shows the cytopathic effects of CN702 and CN764 at various multiplicities of infection on human microvascular endothelial cells.

Figure 4 is a bar graph showing the number of plaque-forming units, expressed as percentage of plaques obtained with wild-type adenovirus, obtained when either LNCaP cells (hatched bars) or HMVEC cells (bars with square pattern) were infected with adenoviral vectors CN739, CN764, CN765 or CN770.

Figure 5 is a line graph illustrating the one-step growth curve of an adenovirus, CN739, in which multiple adenoviral early genes are placed under control of prostate cell specific heterologous TREs, in prostate (LNCaP) and non-prostate cells (HMVEC).

Figure 6 is a line graph illustrating the efficacy in treating a prostate cancer tumor in mice of an adenovirus, CN739, in which multiple adenoviral early genes are placed under control of prostate cell specific heterologous TREs.

Figure 7 is a line graph showing serum PSA levels in mice treated with an adenovirus, CN739, in which multiple adenoviral early genes are placed under control of prostate cell specific heterologous TREs.

Figure 8 (A)-(B) are schematic diagrams of examples of adenovirus vectors in which the E1A and E1B genes are under transcriptional control of cell specific heterologous TREs. Each adenovirus vector contains at least two different heterologous TREs, both of which are functional in the same cell.

Figure 9 depicts schematic diagrams of examples of adenovirus vectors in which the E1A, E1B, and E4 genes are under transcriptional control of cell specific heterologous TREs. Each adenovirus vector contains three different heterologous TREs, all of which are functional in the same cell.

Figure 10 is a graph depicting cytotoxicity of an adenoviral vector containing the coding sequence for adenoviral death protein (ADP), CN751 (solid squares), compared to control CN702 (solid circles), Rec 700 (solid triangles) and mock infection (Xs).

Figure 11 is a graph comparing extracellular virus yield of CN751 (solid squares) and CN702 (solid circles).

Figure 12 is a graph comparing tumor volume in mice harboring LNCaP tumor xenografts challenged with CN751 ("H"), CN702 ("J"), or buffer ("B").

Figure 13 is a schematic depiction of a method for covalent pegylation of an adenovirus. In this method, succinimidyl succinamide is used to covalently attach methoxy-PEG to adenovirus. The pegylated adenovirus is separated from the reaction components by ion exchange chromatography.

Figure 14 shows a half-tone reproduction of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (mobility shift) of pegylated adenovirus proteins. Lanes 1 and 2 are non-pegylated CN706 (control), lanes 3 through 6 are CN706 pegylated under various pH and temperature conditions (lane 3, pH 7.6, room temperature (RT); lane 4, pH 7.6, 4°C; lane 5, pH 8.2, RT; lane 6, pH 8.2, 4°C).

Figure 15 is a chromatogram of ion exchange chromatography analysis of pegylated adenovirus (PEG-706) mixed with control adenovirus CN706.

MODES FOR CARRYING OUT THE INVENTION

We have discovered and constructed replication-competent adenovirus vectors containing heterologous cell-specific transcriptional regulatory elements (TREs) which can

preferentially replicate in cells that allow function of said TREs and have developed methods of using these adenovirus vectors. The adenovirus vectors of this invention comprise a first adenovirus gene under the transcriptional control of a cell-specific heterologous TRE and at least one other gene, such as an adenoviral gene or a transgene, under control of another heterologous TRE which is different from the first TRE, where the heterologous TREs are functional in the same cell but are not the same in polynucleotide sequence (i.e., have different polynucleotide sequences). Preferably, at least two of the heterologous TREs in an adenovirus vector are cell specific for the same cell. Preferably, the adenovirus gene is one that enhances cell death, more preferably one that is essential for adenovirus replication. Preferably, at least one of the adenovirus genes necessary for cell replication is an early gene. Preferably, the genes under transcriptional control of the heterologous TREs are necessary for replication. By providing for cell-specific transcription through the use of multiple heterologous TREs, the invention provides adenovirus vectors that can be used for cell-specific cytotoxic effects due to selective replication.

The adenovirus vectors of the invention replicate preferentially in TRE functional cells (i.e., at a higher yield than in TRE non-functional cells). This replication preference is indicated by comparing the level of replication (i.e., titer) in cells in which the TRE is active to the level of replication in cells in which the TRE is not active. The replication preference is even more significant, as the adenovirus vectors of the invention actually replicate at a significantly lower rate in TRE non-functional cells than wild type virus. Comparison of the adenovirus titer of a TRE active cell type to the titer of a TRE inactive cell type provides a key indication that the overall replication preference is enhanced due to the replication in TRE active cells as well as depressed replication in TRE inactive cells. This is especially useful in the cancer context, in which targeted cell killing is desirable.

The adenovirus vectors of this invention, with the inclusion of at least two different heterologous TREs, are more stable and provide even more cell specificity with regard to replication than previously described adenovirus vectors. Adenovirus vectors have been constructed in which each of the E1A and E1B genes have been placed under transcriptional control of two different heterologous TREs, for example, TREs from the *PSA* gene (*PSE-TRE*) and the probasin gene (*PB-TRE*) and TREs from the *PSA* gene and the *hKLK2* gene (*hKLK2-TRE*).

We have found, for example, that adenoviruses containing the *PSE*-TRE and the *PB*-TRE and adenovirus containing the *PSE*-TRE and the *hKLK2*-TRE appear to possess a stable genome, exhibit higher levels of cell specificity with regard to replication than CN706, an adenovirus with the *PSE*-TRE controlling the E1A gene, and replicate as efficiently as CN706 in prostate cells. As shown herein, in vitro and in vivo results indicate the CN739 (adenovirus with a *PB*-TRE controlling the E1A gene and a *PSE*-TRE controlling the E1B gene, described below) and CN764 (adenovirus with a *PSE*-TRE controlling the E1A gene and an *hKLK2*-TRE controlling the E1B gene, described below) have a 10 to 100-fold higher cell specificity in replication than CN706, while retaining similar prostate tumor killing capacity. Thus, this invention provides an even more attenuated replication competent virus by controlling replication genes with two different heterologous, cell-specific TREs.

Previous attempts to achieve this level of specificity through the construction of adenovirus vectors with the same heterologous TRE controlling transcription of two adenoviral vector genes appear to have resulted in unstable genomes and undesirable polynucleotide sequence rearrangements. Examination of such adenoviruses, with techniques such as restriction enzyme digestion and Southern blot analysis, revealed alterations in the size of adenoviral polynucleotide fragments after viral replication. Without wishing to be bound by theory, such genome instability may be the result of homologous recombination through the duplicated TRE sequences.

The adenovirus vectors provided by the present invention, in which two different heterologous TREs are used to control replication, achieve a stability of the viral genome and an even higher level of target cell specificity than previously described adenoviruses.

The invention uses and takes advantage of what has been considered an undesirable aspect of adenoviral vectors, namely, their replication and possible concomitant immunogenicity. Runaway infection is prevented due to the cell-specific requirements for viral replication. Without wishing to be bound by any particular theory, the inventors note that production of adenovirus proteins can serve to activate and/or stimulate the immune system, either generally or specifically toward target cells producing adenoviral proteins which can be an important consideration in the cancer context, where patients are often moderately to severely immunocompromised.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of
5 the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for
10 Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

For techniques related to adenovirus, see, inter alia, Felgner and Ringold (1989)
15 *Nature* 337:387-388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003-6020; Graham (1984) *EMBO J.* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

Definitions

20 As used herein, "adenovirus" refers to the virus itself or derivatives thereof. The term covers all serotypes and subtypes and both naturally occurring and recombinant forms, except where indicated otherwise.

The term "polynucleotide" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides. Thus, this term includes single-,
25 double- and triple-stranded DNA, as well as single- and double-stranded RNA, RNA-DNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups.
30 Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate (P-NH₂) or a mixed phosphoramidate- phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8; Chaturvedi et al. (1996) *Nucleic*

Acids Res. 24: 2318-23; Schultz et al. (1996) *Nucleic Acids Res.* 24: 2966-73. A phosphorothioate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) *J. Immunol.* 141: 2084-9; Latimer et al. (1995) *Mol. Immunol.* 32: 1057-1064. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. In addition, a double-stranded polynucleotide can be obtained from the single-stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The term "gene" is well understood in the art and is a polynucleotide encoding a polypeptide. In addition to the polypeptide coding regions, a gene includes non-coding regions including, but not limited to, introns, transcribed but untranslated segments, and regulatory elements upstream and downstream of the coding segments.

As used herein, a "transcriptional regulatory element", or "TRE" is a polynucleotide sequence, preferably a DNA sequence, that regulates (i.e., controls) transcription of an operably-linked polynucleotide sequence by an RNA polymerase to form RNA. As used herein, a TRE increases transcription of an operably linked polynucleotide sequence in a host cell that allows the TRE to function. The TRE comprises an enhancer element and/or promoter element, which may or may not be derived from the same gene. The promoter and enhancer components of a TRE may be in any orientation and/or distance from the coding sequence of interest, and comprise multimers of the foregoing, as long as the desired transcriptional activity is obtained. As discussed herein, a TRE may or may not lack a silencer element.

An "enhancer" is a term well understood in the art and is a polynucleotide sequence derived from a gene which increases transcription of a gene which is operably-linked to a promoter to an extent which is greater than the transcription activation effected by the promoter itself when operably-linked to the gene, i.e. it increases transcription from the promoter. Having "enhancer activity" is a term well understood in the art and means what has been stated, i.e., it increases transcription of a gene which is operably linked to a promoter to an extent which is greater than the increase in transcription effected by the

promoter itself when operably linked to the gene, i.e., it increases transcription from the promoter.

A polynucleotide or polynucleotide region has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania).

In the context of adenovirus vector(s), a first heterologous TRE is "different" from a second (or another) heterologous TRE when the polynucleotide sequence identity between the two heterologous TREs is less than about 95%, preferably less than about 90%, preferably less than about 85%, preferably less than about 75%. Generally, "different TREs" are derived from the transcriptional regulatory regions of different genes. "Different TREs" may also be derived from the transcriptional regulatory region of the same gene, as long as the sequence identity between them is less than the values listed above (i.e., less than about 95%, preferably less than about 90%, preferably less than about 85%, preferably less than about 75%). Although two different TREs are not identical in polynucleotide sequence, they may be functional in the same cell and may also have the same cell-specificity.

As used herein, a TRE derived from a specific gene is referred to by the gene from which it was derived and is a polynucleotide sequence which regulates transcription of an operably linked polynucleotide sequence in a host cell that expresses said gene. For example, as used herein, a "human glandular kallikrein transcriptional regulatory element", or "*hKLK2*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows an *hKLK2*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor. An *hKLK2*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of an *hKLK2* promoter and/or an *hKLK2* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a “probasin (*PB*) transcriptional regulatory element”, or “*PB*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably-linked polynucleotide sequence in a host cell that allows a *PB*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor. A *PB*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PB* promoter and/or a *PB* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a “prostate-specific antigen (*PSA*) transcriptional regulatory element”, or “*PSA*-TRE”, or “*PSE*-TRE” is polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *PSA*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor. A *PSE*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PSA* promoter and/or a *PSA* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a “carcinoembryonic antigen (*CEA*) transcriptional regulatory element”, or “*CEA*-TRE” is polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *CEA*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses CEA. The *CEA*-TRE is responsive to transcription factors and/or co-factor(s) associated with CEA-producing cells and comprises at least a portion of the *CEA* promoter and/or enhancer.

As used herein, an “ α -fetoprotein (*AFP*) transcriptional regulatory element”, or “*AFP*-TRE” is polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably linked polynucleotide sequence) in a host cell that allows an *AFP*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses AFP. The *AFP*-TRE is responsive to transcription factors and/or co-factor(s) associated with AFP-producing cells and comprises at least a portion of the *AFP* promoter and/or enhancer.

As used herein, an “a mucin gene (*MUC*) transcriptional regulatory element”, or “*MUC1*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably-linked polynucleotide sequence) in a host cell that allows an *MUC1*-TRE to function, such as a cell (preferably a mammalian

cell, even more preferably a human cell) that expresses MUC1. The *MUC1*-TRE is responsive to transcription factors and/or co-factor(s) associated with MUC1-producing cells and comprises at least a portion of the *MUC1* promoter and/or enhancer.

5 A "cell-specific TRE" is preferentially functional in a specific type of cell relative to other types of cells of different functionality. A cell-specific TRE may or may not be tumor cell specific.

As used herein, the term "target cell-specific" is intended to mean that the TRE sequences to which a gene essential for replication of an adenoviral vector is operably linked, or to which a transgene is operably linked, functions specifically in that target cell so that replication proceeds in that target cell, or so that a transgene polynucleotide is expressed in that target cell. This can occur by virtue of the presence in that target cell, and not in non-target cells, of transcription factors that activate transcription driven by the operably linked transcriptional control sequences. It can also occur by virtue of the absence of transcription inhibiting factors that normally occur in non-target cells and prevent transcription driven by the operably linked transcriptional control sequences. The term "target cell-specific", as used herein, is intended to include cell type specificity, tissue specificity, as well as specificity for a cancerous state of a given target cell. In the latter case, specificity for a cancerous state of a normal cell is in comparison to a normal, non-cancerous counterpart.

20 The activity of a TRE generally depends upon the presence of transcriptional regulatory factors and/or the absence of transcriptional regulatory inhibitors. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operatively linked to) the TRE. As discussed herein, a TRE can be of varying lengths, and of varying sequence composition. By transcriptional activation, it is intended that transcription will be increased above basal levels in the target cell by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold. More preferably at least about 50-fold, more preferably at least about 100-fold, even more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably, at least about 1000-fold. Basal levels are generally the level of activity, if any, in a non-target cells, or the level of activity (if any) of a reporter construct lacking the TRE of interest as tested in a target cell type.

A "functionally-preserved" variant of a TRE is a TRE which differs from another TRE, but still retains ability to increase transcription of an operably linked polynucleotide, especially cell-specific transcription activity. The difference in a TRE can be due to differences in linear sequence, arising from, for example, single or multiple base
5 mutation(s), addition(s), deletion(s), and/or modification(s) of the bases. The difference can also arise from changes in the sugar(s), and/or linkage(s) between the bases of a TRE.

Certain point mutations within sequences of TREs have been shown to decrease transcription factor binding and gene activation. One of skill in the art would recognize that some alterations of bases in and around known the transcription factor binding sites
10 are more likely to negatively affect gene activation and cell-specificity, while alterations in bases which are not involved in transcription factor binding are not as likely to have such effects. Certain mutations are also capable of increasing TRE activity. Testing of the effects of altering bases may be performed *in vitro* or *in vivo* by any method known in the art, such as mobility shift assays, or transfecting vectors containing these alterations in
15 TRE functional and TRE non-functional cells. Additionally, one of skill in the art would recognize that point mutations and deletions can be made to a TRE sequence without altering the ability of the sequence to regulate transcription.

"Under transcriptional control" is a term well-understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its
20 being operably (operatively) linked to an element which contributes to the regulation of, either promotes or inhibits, transcription.

The term "operably linked" relates to the orientation of polynucleotide elements in a functional relationship. A TRE is operably linked to a coding segment if the TRE promotes transcription of the coding sequence. Operably linked means that the DNA
25 sequences being linked are generally contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable length, some polynucleotide elements may be operably linked but not contiguous.

As used herein, "a cell which allows a TRE to function" or a cell in which the function of a TRE is "sufficiently preserved", or "a cell in which a TRE is functional" is a cell in which the TRE, when operably linked to a promoter (if not included in the TRE) and a reporter gene, increases expression of the reporter gene at least about 2-fold,
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preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably at least about 1000-fold, when compared to the expression of the same promoter and reporter gene when not operably linked to said TRE. Methods for measuring levels (whether relative or absolute) of expression are known in the art and are described herein.

A "target cell" is any cell that allows a heterologous TRE to function. Preferably, a target cell is a mammalian cell, more preferably a human cell.

As used herein, "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer", and "cancer cells" (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. Neoplastic cells can be benign or malignant.

"Androgen receptor", or AR as used herein refers to a protein whose function is to specifically bind to androgen and, as a consequence of the specific binding, recognize and bind to an androgen response element (ARE), following which the AR is capable of regulating transcriptional activity. The AR is a nuclear receptor that, when activated, binds to cellular androgen-responsive element(s). In normal cells the AR is activated by androgen, but in non-normal cells (including malignant cells) the AR may be activated by non-androgenic agents, including hormones other than androgens. Encompassed in the term "androgen receptor" are mutant forms of an androgen receptor, as long as the function is sufficiently preserved. Mutants include androgen receptors with amino acid additions, insertions, truncations and deletions, as long as the function is sufficiently preserved. In this context, a functional androgen receptor is one that binds both androgen and, upon androgen binding, an ARE.

An "adenovirus vector" or "adenoviral vector" (used interchangeably) is a term well understood in the art and generally comprises a polynucleotide (defined herein) comprising all or a portion of an adenovirus genome. For the purpose of the present invention, an adenovirus vector contains a heterologous TRE operably linked to a polynucleotide. The operably linked polynucleotide can be adenoviral or heterologous. An adenovirus is exemplified by, but not limited to, Ad2, Ad5, Ad12, and Ad40. The terms "vector", "polynucleotide vector", "construct", "polynucleotide construct" and "vector construct" are used interchangeably herein. An adenoviral vector of this invention

may be in any of several forms, including, but not limited to, naked DNA, DNA encapsulated in an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), DNA encapsulated in liposomes, DNA complexed with polylysine, complexed with synthetic polycationic molecules, conjugated with transferrin, complexed with compounds such as PEG to immunologically “mask” the molecule and/or increase half-life, or conjugated to a non-viral protein. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

“Heterologous” means derived from a genotypically distinct entity from that of the rest of the entity to which it is being compared.

A “heterologous” TRE is one which is not associated with or derived from a wild-type adenovirus. Examples of heterologous TREs are the albumin promoter or enhancer and other viral promoters and enhancers, such as SV40. Examples of preferred heterologous TREs are provided herein.

A “heterologous gene” or “transgene” is any gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target cell prior to introduction by the adenovirus vector. Examples of preferred transgenes are provided below.

An “endogenous” promoter, enhancer, or TRE is native to or derived from adenovirus.

“Replication” and “propagation” are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce or proliferate. This term is well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay or plaque assay. “Replication” and “propagation” include any activity directly or indirectly involved in the process of virus manufacture, including, but not limited to, viral gene expression, production of viral proteins, nucleic acids or other components, packaging of viral components into complete viruses, and cell lysis.

A “gene essential for replication” is a gene whose transcription is required for the vector to replicate in a cell.

The terms “polypeptide”, “peptide” and “protein” are used interchangeably to refer to polymers of amino acids of any length. These terms also include proteins that are post-

translationally modified through reactions that include glycosylation, acetylation and phosphorylation.

5 A "host cell" includes an individual cell or cell culture which can be or has been a recipient of an adenoviral vector of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected or infected *in vivo* with an adenoviral vector of this invention.

10 As used herein, "cytotoxicity" is a term well understood in the art and refers to a state in which one or more of a cell's usual biochemical or biological functions are aberrantly compromised (i.e., inhibited or elevated). These activities include, but are not limited to, metabolism, cellular replication, DNA replication, transcription, translation, and uptake of molecules. "Cytotoxicity" includes cell death and/or cytolysis. Assays are known in the art which indicate cytotoxicity, such as dye exclusion, ³H-thymidine uptake, and plaque assays. The term "selective cytotoxicity", as used herein, refers to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which allows a TRE to function when compared to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which does not allow, or is less permissive for, the same TRE to function. Such cytotoxicity may be measured, for example, by plaque assays, reduction or stabilization in size of a tumor comprising target cells, or the reduction or stabilization of serum levels of a marker characteristic of the tumor cells or a tissue-specific marker, e.g., a cancer marker such as prostate specific antigen.

20 As used herein, a "cytotoxic" gene is a gene whose expression in a cell, either alone or in conjunction with adenovirus replication, enhances the degree and/or rate of cytotoxic and/or cytolytic activity in the cell.

25 A "therapeutic" gene is a gene whose expression in a cell is associated with a desirable result. In the cancer context, this desirable result may be, for example, cytotoxicity, repression or slowing of cell growth, and/or cell death.

30 A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom and the progeny thereof. The definition also includes samples that have been manipulated in any way after their

procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

5 An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, and pets.

 An "effective amount" is an amount sufficient to effect beneficial or desired clinical results. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of an adenoviral vector is an amount
10 that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

 As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease,
15 stabilized (i.e., not worsening) state of disease, preventing spread (i.e., metastasis) of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

20 A "masked adenovirus" is an adenovirus which has been complexed with a hydrophilic polymer ("masking agent"). The adenovirus may be any adenovirus, including naturally occurring isolates of adenovirus or engineered adenovirus vectors such as those disclosed in the instant application. Masking agents are preferably of low immunogenicity. Examples of acceptable hydrophilic polymers include:
25 polyethylene/polypropylene copolymers, polyacrylic acid analogues, sugar polymers such as cellulose, polyformaldehyde, poly(N-vinylpyrrolidone), polyethylene glycol (PEG), and the like. The hydrophilic polymer may be complexed by covalent or non-covalent attachment to the capsid proteins of the virus, particularly the hexon and fiber capsid proteins. A preferred hydrophilic polymer is PEG, and a preferred masked adenovirus is
30 PEG covalently linked to adenovirus ("covalently pegylated adenovirus").

 "Palliating" a disease means that the extent and/or undesirable clinical manifestations of a disease state are lessened and/or time course of the progression is

slowed or lengthened, as compared to not administering adenoviral vectors of the present invention.

Adenoviral vectors comprising heterologous TREs

5 The present invention provides adenoviral vectors which comprise a first adenovirus gene under transcriptional control of a heterologous (i.e., non-adenovirus) TRE and at least a second gene under transcriptional control of a second heterologous TRE, wherein the two heterologous TREs are different from each other (in nucleotide sequence) but are functional in the same cell. In addition, at least the first heterologous TRE is cell-specific. Preferably, the adenovirus gene is one that contributes to cytotoxicity (whether
10 direct and/or indirect), more preferably one that contributes to and/or causes cell death, and even more preferably the first adenoviral gene is essential from adenovirus replication. Examples of an adenovirus gene that contributes to cytotoxicity include, but are not limited to, the adenovirus death protein gene. See Fig.1 for diagrammatic examples.

15 Because the adenovirus vector(s) is selectively (i.e. preferentially) replication-competent for propagation in target cells which allow the function of the heterologous TREs, these cells will be preferentially subject to the cytotoxic and/or cytolytic effects of adenoviral proliferation. Preferably, target cells are neoplastic cells, although any cell for which it is desirable and/or tolerable to sustain this cytotoxic activity may be a target cell.
20 By combining the adenovirus vector(s) with the mixture of target and non-target cells, *in vitro* or *in vivo*, the adenovirus vector(s) preferentially replicates in the target cells. Once the target cells are destroyed due to selective cytotoxic and/or cytolytic replication, the adenovirus vector(s) replication is significantly reduced, lessening the probability of runaway infection and undesirable bystander effects. *In vitro* cultures may be retained to
25 continually monitor the mixture (such as, for example, a biopsy or other appropriate biological sample) for occurrence (i.e. presence) and/or recurrence of the target cell, e.g., a cancer cell in which the cell specific TRE is functional.

 To ensure cytotoxicity further, one or more transgenes having a cytotoxic effect may also be present and under the selective transcriptional control of a heterologous TRE.
30 Additionally, or alternatively, an adenovirus gene that contributes to cytotoxicity and/or cell death (such as the adenovirus death protein (ADP) gene) may be included in the adenoviral vector, either free of, or under, the selective transcriptional control of a

heterologous TRE. In these embodiments, one may provide higher confidence that the target cells will be destroyed.

5 In one embodiment, the invention provides an adenovirus vector comprising an adenovirus gene essential for replication under transcriptional control of a heterologous, cell specific TRE, wherein the TRE selectively regulates the expression of said gene in a target cell. Further, the adenovirus vector comprises a second gene under transcriptional control of a second heterologous TRE, wherein the first and second heterologous TREs are functional in the same cell and the TREs are different from each other.

10 Cell, or tissue, specific transcriptional regulatory elements are well known in the art. TREs may be derived from the transcriptional regulatory sequences of a single gene or from different genes and combined to produce a functional TRE. A cell-specific TRE is preferentially functional in a limited population (or type) of cells, e.g., prostate cells or liver cells.

15 As is known in the art, activity of TREs can be inducible. Inducible TREs generally exhibit low activity in the absence of the inducer, and are up-regulated in the presence of the inducer. Inducible TREs may be preferred when expression is desired only at certain times or at certain locations, or when it is desirable to titrate the level of expression using an inducing agent. For example, transcriptional activity from the *PSE*-TRE, *PB*-TRE and *hKLK2*-TRE is inducible by androgen, as described herein.

20 Accordingly, in one embodiment, a heterologous TRE of the adenovirus vector is inducible.

As mentioned, TRE can also comprise multimers. For example, a TRE can comprise a tandem series of at least two, at least three, at least four, at least five promoter fragments. Alternatively, a TRE could have one or more promoter regions along with one or more enhancer regions. These multimers may also contain promoter and/or enhancer sequences from different genes. In addition, the promoter and enhancer components of a TRE may be in any orientation and/or distance from the coding sequence of interest, as long as the desired cell-specific transcriptional activity is obtained.

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A TRE used in the present invention may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element known in the art) can assist in shutting off transcription (and thus replication) in non-permissive cells. Thus, presence of a silencer can confer enhanced cell-specific replication by more effectively preventing adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in

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effecting replication in target cells, thus conferring enhanced cell-specific replication due to more effective replication in target cells.

When used in an adenovirus vector of the present invention, the heterologous TREs have a different polynucleotide sequence from each other. Accordingly, in a given
5 adenovirus vector, the sequence identity between the two heterologous TREs is less than about 95%, preferably less than about 90%, more preferably less than about 85%, more preferably less than 80%, even more preferably less than about 75%. Despite the difference in sequence identity, the heterologous TREs of a given adenovirus vector are functional in the same cell. The difference(s) may arise from, for example, varying the
10 sequence of a TRE derived from a single gene. It is possible to generate such TREs using standard methods in the art and/or allowing sequences to alter during the course of propagation. The difference(s) may also arise from TREs that are derived from different genes.

As is readily appreciated by one skilled in the art, a TRE is a polynucleotide
15 sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion are known in the art, and readily available functional assays (such as the CAT or luciferase reporter gene assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite cell-specific transcription function.

20 Hence, functionally preserved variants of TREs may also be used including nucleic acid substitutions, additions, and/or deletions. Accordingly, variants of TREs must retain function in the target cell but need not be of maximal function.

It is possible that certain base modifications will result in enhanced expression levels and/or cell-specificity. Nucleic acid sequence deletions or additions within a TRE
25 may decrease or increase transcription if they bring transcription regulatory protein binding sites too close or too far away or rotate them so they are on opposite sides of the DNA helix, as is known in the art. Thus, while the inventors are not wishing to be bound by a single theory, it is possible that certain modifications will result in modulated resultant expression levels, including enhanced cell-specific expression levels.

30 Achievement of enhanced expression levels may be especially desirable in the case of more aggressive forms of neoplastic growth, or when a more rapid and/or aggressive pattern of cell killing is warranted (due to an immunocompromised condition of the individual, for example).

Transcriptional activity (including decrease or enhancement) can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operably linked to) a TRE. As discussed herein, a TRE can be of varying lengths, and of varying sequence composition. By transcriptional activity, it is intended that transcription due to the presence of the enhancer is increased above basal levels (i.e., promoter alone; lacking enhancer) in the target cell by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably at least about 1000-fold. Basal levels are generally the level of activity, if any, in a non-target cell, or the level of activity (if any) of a reporter construct lacking a TRE as tested in a non-target cell.

Maximal transcriptional activation activity of a TRE may not always be necessary to achieve a desired result. The level of induction afforded by a fragment of a TRE may be sufficient in certain applications to achieve a desired result. For example, if used for treatment or palliation of a disease state, less-than-maximal responsiveness may be sufficient for the desired result, if, for example, the target cells are not especially virulent and/or the extent of disease is relatively confined.

The size of the heterologous TREs will be determined in part by the capacity of the adenoviral vector, which in turn depends upon the contemplated form of the vector (see below). Generally minimal sizes are preferred, as this provides potential room for insertion of other sequences which may be desirable, such as transgenes (discussed below) or other additional regulatory sequences. However, if no additional sequences are contemplated, or if, for example, an adenoviral vector will be maintained and delivered free of any viral packaging constraints, larger DNA sequences may be used as long as the resultant adenoviral vector is rendered replication-competent.

If no adenovirus sequences have been deleted, an adenoviral vector can be packaged with extra sequences totaling up to about 5% of the genome size, or approximately 1.8 kb. If non-essential sequences are removed from the adenovirus genome, an additional 4.6 kb of insert can be tolerated (i.e., a total of about 1.8 kb plus 4.6 kb, which is about 6.4 kb). Examples of non-essential adenoviral sequences that can be deleted are E3 and E4 (as long as the E4 ORF6 is maintained).

In order to minimize non-specific replication, endogenous (i.e., adenovirus) TREs should preferably be removed. This would also provide more room for inserts in an adenoviral vector, which may be of special concern if an adenoviral vector will be packaged as a virus (see below). Even more importantly, deletion of endogenous TREs would prevent a possibility of a recombination event whereby a heterologous TRE is deleted and the endogenous TRE assumes transcriptional control of its respective adenovirus coding sequences (thus allowing non-specific replication). In one embodiment, an adenoviral vector of the invention is constructed such that the endogenous transcription control sequences of adenoviral genes are deleted and replaced by a heterologous TREs. However, endogenous TREs may be maintained in the adenovirus vector(s), provided that sufficient cell-specific replication preference is preserved. These embodiments can be constructed by providing heterologous TREs intervening between the endogenous TRE and the replication gene coding segment. Requisite cell-specific replication preference is indicated by conducting assays that compare replication of the adenovirus vector in a cell which allows function of the heterologous TREs with replication in a cell which does not.

Generally, it is intended that replication is increased above basal levels in the target cell by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500- fold, even more preferably at least about 1000-fold. The acceptable differential can be determined empirically (using, for example, Northern assays or other known in the art) and will depend upon the anticipated use of the adenoviral vector and/or the desired result.

(a) Exemplary heterologous TREs

Cell, or tissue, specific transcriptional regulatory elements are well known in the art. Methods to identify such elements are also well known in the art. The cell specific TREs provided below are illustrative examples and not meant to limit the invention.

In one embodiment, the invention includes adenovirus vectors wherein the heterologous TREs are prostate cell specific. For example, TREs that function preferentially in prostate cells and can be used in the present invention to target adenovirus replication to prostate neoplasia, include, but are not limited to, TREs derived from the

prostate-specific antigen gene (*PSA*-TRE), the glandular kallikrein-1 gene (from the human gene, *hKLK2*-TRE), and the probasin gene (*PB*-TRE). All three of these genes are preferentially expressed in prostate cells and the expression is androgen-inducible. Generally, expression of genes responsive to androgen induction requires the presence of an androgen receptor (AR).

PSA is synthesized exclusively by normal, hyperplastic, and malignant prostatic epithelia; hence, its tissue-specific expression has made it an excellent biomarker for benign prostatic hyperplasia (BPH) and prostatic carcinoma (CaP). Normal serum levels of PSA are typically below 5 ng/ml, with elevated levels indicative of BPH or CaP.

Lundwall et al. (1987) *FEBS Lett.* 214: 317; Lundwall (1989) *Biochem. Biophys. Res. Comm.* 161: 1151; and Riegmann et al. (1991) *Molec. Endocrin.* 5: 1921.

The region of the *PSA* gene that is used to provide cell specificity dependent upon androgens, particular in prostate cells, involves approximately 6.0 kilobases. Schuur et al. (1996) *J. Biol. Chem.* 271:7043-7051. An enhancer region of approximately 1.5 kb in humans is located between nt -5322 and nt -3739, relative to the transcription start site of the *PSA* gene. The *PSA* promoter consists of the sequence from about nt -540 to nt +8 relative to the transcription start site. Juxtapositioning of these two genetic elements yield a fully functional, minimal prostate-specific enhancer/promoter (*PSE*) TRE. Other portions of the approximately 6.0 kb region of the *PSA* gene can be used in the present invention, as long as requisite functionality is maintained.

The *PSE* and *PSA* TRE depicted in (SEQ ID NO:1) is the same as that given in GenBank Accession No. U37672, and published. Schuur et al. (1996). A variant *PSA*-TRE nucleotide sequence is depicted in (SEQ ID NO:2). This is the *PSA*-TRE contained within CN706 clone 35.190.13. CN706 is an adenoviral vector in which the E1A gene in Ad5 is under transcriptional control of a *PSA*-TRE. CN706 demonstrates selective cytotoxicity toward PSA-expressing cells *in vitro* and *in vivo*. Rodriguez et al. (1997). CN706 was passaged through 293 and LNCaP cells. A clone, designated 35.190.13 was isolated. The structure of this clone was confirmed by PCR, restriction endonuclease digestion and Southern blotting. Both DNA strands of the CN706 clone 35.190.13 were sequenced between positions 1 and 3537. Seven single base pair changes were found in the *PSE*, compared to the sequence reported by Schuur et al. (1996). These point mutations are not in the ARE and are thus not likely to affect the function of the enhancer. One mutation was found in the *PSA* promoter region, but is not likely to affect gene

expression from this promoter. In addition to these mutations, a missense mutation was found in the first exon of E1A. This C to G transition at position 3032 results in a Glu to Arg change in the E1A protein sequence. This mutation does not appear to diminish E1A function.

5 Human glandular kallikrein (*hKLK2*, encoding the hK2 protein) is expressed exclusively in the prostate and its expression is up-regulated by androgens primarily by transcriptional activation. Wolf et al. (1992) *Molec. Endocrinol.* 6:753-762. Morris (1989) *Clin. Exp. Pharm. Physiol.* 16:345-351; Qui et al. (1990) *J. Urol.* 144:1550-1556; Young et al. (1992) *Biochem.* 31:818-824. The levels of hK2 found in various tumors and
10 in the serum of patients with prostate cancer differ substantially from those of PSA and indicate that hK2 antigen may be a significant marker for prostate cancer. Circulating hK2 in different relative proportions to PSA has been detected in the serum of patients with prostate cancer. Charlesworth et al. (1997) *Urology* 49:487-493. Expression of hK2 has been detected in each of 257 radical prostatectomy specimens analyzed. Darson et al.
15 (1997) *Urology* 49:857-862. The intensity and extent of hK2 expression, detected using specific antibodies, increased from benign epithelium to high-grade prostatic intraepithelial neoplasia (PIN) and adenocarcinoma, whereas PSA and prostate acid phosphatase displayed an inverse pattern of immunoreactivity. Darson et al. (1997). Indeed, it has been reported that a certain percentage of PSA-negative tumors have
20 detectable hK2. Tremblay et al. (1997) *Am. J. Pathol.* 150:455-459.

The activity of the *hKLK2* 5' promoter has been previously described and a region up to -2256 relative to the transcription start site was previously disclosed. Schedlich et al. (1987) *DNA* 6:429-437. The *hKLK2* promoter is androgen responsive and, in plasmid constructs wherein the promoter alone controls the expression of a reporter gene,
25 expression of the reporter gene is increased approximately 10-fold in the presence of androgen. Murtha et al. (1993) *Biochem.* 32:6459-6464. *hKLK2* enhancer activity is found within a polynucleotide sequence approximately nt -12,014 to nt -2257 relative to the start of transcription (depicted in SEQ ID NO:3) and, when this sequence is operably linked to an *hKLK2* promoter and a reporter gene, transcription of operably-linked
30 sequences in prostate cells increases in the presence of androgen at levels approximately 30- to approximately 100-fold over the level of transcription in the absence of androgen. This induction is generally orientation independent and position independent. Enhancer activity has also been demonstrated in the following regions (all relative to the

transcription start site): about nt -3993 to about nt -3643 (nt 8021 to 8371 of SEQ ID NO:3), about nt -4814 to about nt -3643 (nt 7200 to 8371 of SEQ ID NO:3), about nt -5155 to about nt -3387 (nt 6859 to 8627 of SEQ ID NO:3), about nt -6038 to about nt -2394 (nt 5976 to 9620 of SEQ ID NO:3).

5 Thus, an *hKLK2* enhancer can be operably linked to an *hKLK2* promoter or a heterologous promoter to form an *hKLK2* transcriptional regulatory element (*hKLK2*-TRE). An *hKLK2*-TRE can then be operably linked to a heterologous polynucleotide to confer *hKLK2*-TRE-specific transcriptional regulation on the linked gene, thus increasing its expression.

10 The rat probasin (*PB*) gene encodes a nuclear and secreted protein, probasin, that is only expressed in the dorsolateral prostate. Dodd et al. (1983) *J. Biol. Chem.* 258:10731-10737; Matusik et al. (1986) *Biochem. Cell. Biol.* 64: 601-607; and Sweetland et al. (1988) *Mol. Cell. Biochem.* 84: 3-15. The dorsolateral lobes of the murine prostate are considered the most homologous to the peripheral zone of the human prostate, where approximately
15 68% of human prostate cancers are thought to originate.

A *PB*-TRE has been shown in an approximately 0.5 kb fragment of sequence upstream of the probasin coding sequence, from about nt -426 to about nt +28 relative to the transcription start site, as depicted in (SEQ ID NO:4). This minimal promoter sequence from the *PB* gene appears to provide sufficient information to direct development and
20 hormone-regulated expression of an operably linked heterologous gene specifically to the prostate in transgenic mice. Greenberg et al. (1994) *Mol. Endocrinol.* 8:230-239.

Thus, TREs derived from prostate cell specific TREs, including, but not limited to, those described herein, may be used in the present invention to generate stable adenovirus vectors that preferentially replicate in cells in which the TREs are functional, such as cells
25 expressing an AR, particularly cells derived from prostate neoplasia. Accordingly, and by way of example, the invention includes adenovirus vectors in which the first heterologous TRE is a *PSA*-TRE (e.g., *PSE*-TRE) and the second heterologous TRE is a *PB*-TRE, in which the first heterologous TRE is a *PB*-TRE and the second heterologous TRE is a *PSA*-TRE (e.g., *PSE*-TRE), in which the first heterologous TRE is an *hKLK2*-TRE and the
30 second heterologous TRE is a *PSA*-TRE (e.g., *PSE*-TRE), in which the first heterologous TRE is a *PSA*-TRE (e.g., *PSE*-TRE) and the second heterologous TRE is an *hKLK2*-TRE, in which the first heterologous TRE is an *hKLK2*-TRE and the second heterologous TRE

is a *PB*-TRE, in which the first heterologous TRE is a *PB*-TRE and the second heterologous TRE is an hKLK2-TRE, as described above. See Examples 1-4, Fig. 2.

In the present invention, replication-competent adenovirus vectors directed at specific target cells may also be generated with the use of TREs that are preferentially functional in the target tumor cells. Non-limiting examples of tumor cell-specific heterologous TREs, and non-limiting examples of respective potential target cells, include TREs from the following genes: α -fetoprotein (*AFP*) (liver cancer), mucin-like glycoprotein DF3 (*MUC1*) (breast carcinoma), carcinoembryonic antigen (*CEA*) (colorectal, gastric, pancreatic, breast, and lung cancers), plasminogen activator urokinase (*uPA*) and its receptor gene (breast, colon, and liver cancers), *E2F1* (cell cycle S-phase specific promoter) (tumors with disrupted retinoblastoma gene function), *HER-2/neu* (*c-erbB2/neu*) (breast, ovarian, stomach, and lung cancers).

In the present invention, tumor-specific TREs may be used in conjunction with tissue-specific TREs from the following exemplary genes (tissue in which the TREs are specifically functional are in parentheses): hypoxia responsive element, vascular endothelial growth factor receptor (endothelium), albumin (liver), factor VII (liver), fatty acid synthase (liver), Von Willebrand factor (brain endothelium), alpha-actin and myosin heavy chain (both in smooth muscle), synthetast I (small intestine), Na-K-Cl transporter (kidney). Additional tissue specific TREs are known in the art.

Accordingly, in one embodiment, the cell specific, heterologous TRE is tumor cell specific. Preferably, both heterologous TREs are tumor cell specific and functional in the same cell. In another embodiment, one of the first heterologous TREs is tumor cell specific and the second heterologous TRE is tissue specific, whereby both TREs are function in the same cell.

AFP is an oncofetal protein, the expression of which is primarily restricted to developing tissues of endodermal origin (yolk sac, fetal liver, and gut), although the level of its expression varies greatly depending on the tissue and the developmental stage. AFP is of clinical interest because the serum concentration of AFP is elevated in a majority of hepatoma patients, with high levels of AFP found in patients with advanced disease. The serum AFP levels in patients appear to be regulated by AFP expression in hepatocellular carcinoma but not in surrounding normal liver. Thus, the AFP gene appears to be regulated to hepatoma cell-specific expression.

Cell-specific TREs from the *AFP* gene have been identified. For example, the cloning and characterization of human AFP-specific enhancer activity is described in Watanabe et al. (1987) *J. Biol. Chem.* 262:4812-4818. The entire 5' *AFP* flanking region (containing the promoter, putative silencer, and enhancer elements) is contained within approximately 5 kb upstream from the transcription start site (SEQ ID NO:5).

The *AFP* enhancer region in human is located between about nt -3954 and about nt -3335, relative to the transcription start site of the *AFP* gene. The human *AFP* promoter encompasses a region from about nt -174 to about nt +29. Juxtapositioning of these two genetic elements, as depicted in SEQ ID NO:6, yields a fully functional *AFP*-TRE. Ido et al. (1995) describe a 259 bp promoter fragment (nt -230 to nt +29) that is specific for HCC. *Cancer Res.* 55:3105-3109. The *AFP* enhancer contains two regions, denoted A and B, located between nt -3954 and nt -3335 relative to the transcription start site. The promoter region contains typical TATA and CAAT boxes. Preferably, the *AFP*-TRE contains at least one enhancer region. More preferably, the *AFP*-TRE contains both enhancer regions.

Suitable target cells for adenoviral vectors containing *AFP*-TREs are any cell type that allow an *AFP*-TRE to function. Preferred are cells that express, or produce, AFP, including, but not limited to, tumor cells expressing AFP. Examples of such cells are hepatocellular carcinoma cells, gonadal and other germ cell tumors (especially endodermal sinus tumors), brain tumor cells, ovarian tumor cells, acinar cell carcinoma of the pancreas (Kawamoto et al. (1992) *Hepatogastroenterology* 39:282-286), primary gall bladder tumor (Katsuragi et al. (1989) *Rinsko Hoshasen* 34:371-374), uterine endometrial adenocarcinoma cells (Koyama et al. (1996) *Jpn. J. Cancer Res.* 87:612-617), and any metastases of the foregoing (which can occur in lung, adrenal gland, bone marrow, and/or spleen). In some cases, metastatic disease to the liver from certain pancreatic and stomach cancers produce AFP. Especially preferred are hepatocellular carcinoma cells and any of their metastases. AFP production can be measured using assays standard in the art, such as RIA, ELISA or Western blots (immunoassays) to determine levels of AFP protein production or Northern blots to determine levels of AFP mRNA production. Alternatively, such cells can be identified and/or characterized by their ability to activate transcriptionally an *AFP*-TRE (i.e., allow an *AFP*-TRE to function).

The protein urokinase plasminogen activator (uPA) and its cell surface receptor, urokinase plasminogen activator receptor (uPAR), are expressed in many of the most

frequently occurring neoplasia and appear to represent important proteins in cancer metastasis. Both proteins are implicated in breast, colon, prostate, liver, renal, lung and ovarian cancer. Transcriptional regulatory elements that regulate uPA and uPAR transcription have been extensively studied. Riccio et al. (1985) *Nucleic Acids Res.* 13:2759-2771; Cannio et al., (1991) *Nucleic Acids Res.* 19:2303-2308.

Thus, cell-specific TREs, including but not limited to those described herein, could be used in the present invention to generate stable adenovirus vectors that preferentially replicate in cells in which the TREs are functional, such as cells derived from liver neoplasia. In one embodiment, the invention includes adenovirus vectors wherein at least one of the heterologous TREs are liver cell-specific. Accordingly, and by way of example, the invention includes adenovirus vectors in which the first heterologous TRE is an *AFP*-TRE and the second heterologous TRE is an *uPA*-TRE, in which the first heterologous TRE is an *uPA*-TRE and the second heterologous TRE is an *AFP*-TRE, in which the first heterologous TRE is an *AFP*-TRE and the second heterologous TRE is an *albumin*-TRE, in which the first heterologous TRE is an *albumin*-TRE and the second heterologous TRE is an *AFP*-TRE, as described above.

CEA is a 180,000-Dalton glycoprotein tumor-associated antigen present on endodermally-derived neoplasia of the gastrointestinal tract, such as colorectal, gastric (stomach) and pancreatic cancer, as well as other adenocarcinomas such as breast and lung cancers. CEA is of clinical interest because circulating CEA can be detected in the great majority of patients with CEA-positive tumors. In lung cancer, about 50% of total cases have circulating CEA, with high concentrations of CEA (greater than 20 ng/ml) often detected in adenocarcinomas. Approximately 50% of patients with gastric carcinoma are serologically positive for CEA.

The 5' upstream flanking sequence of the *CEA* gene has been shown to confer cell-specific activity. The *CEA* promoter region, approximately the first 424 nucleotides upstream of the translational start site in the 5' flanking region of the gene, was shown to confer cell-specific activity when the region provided higher promoter activity in CEA-producing cells than in non-producing HeLa cells. Schrewe et al. (1990) *Mol. Cell. Biol.* 10:2738-2748. In addition, cell-specific enhancer regions have been found. WO/95/14100. The entire 5' *CEA* flanking region (containing the promoter, putative silencer, and enhancer elements) appears to be contained within approximately 14.5 kb upstream from the transcription start site. Richards et al. (1995); WO 95/14100. Further

characterization of the 5' flanking region of the *CEA* gene by Richards et al. (1995) indicated two upstream regions, -13.6 to -10.7 kb or -6.1 to -4.0 kb, when linked to the multimerized promoter resulted in high-level and selective expression of a reporter construct in *CEA*-producing LoVo and SW1463 cells. Richards et al. (1995) also
5 localized the promoter region to nt -90 and nt +69 relative to the transcriptional start site, with region nt -41 to nt -18 as essential for expression. WO95/14100 describes a series of 5' flanking *CEA* fragments which confer cell-specific activity, such as about nt -299 to about nt +69; about nt -90 to about nt +69; nt -14,500 to nt -10,600; nt -13,600 to nt -10,600, nt -6100 to nt -3800. In addition, cell specific transcription activity is conferred on
10 an operably linked gene by the *CEA* fragment from nt -402 to nt +69, depicted in (SEQ ID NO:7). Any *CEA*-TREs used in the present invention are derived from mammalian cells, including but not limited to, human cells. Thus, any of the *CEA*-TREs may be used in the invention as long as requisite desired functionality is displayed in the adenovirus vector. The cloning and characterization of *CEA* sequences have been described in the literature
15 and are thus made available for practice of this invention and need not be described in detail herein.

The protein product of the *MUC1* gene (known as mucin or MUC1 protein; episialin; polymorphic epithelial mucin or PEM; EMA; DF3 antigen; NPGP; PAS-O; or CA15.3 antigen) is normally expressed mainly at the apical surface of epithelial cells
20 lining the glands or ducts of the stomach, pancreas, lungs, trachea, kidney, uterus, salivary glands, and mammary glands. Zotter et al. (1988) *Cancer Rev.* 11-12: 55-101; and Girling et al. (1989) *Int. J. Cancer* 43: 1072-1076. However, mucin is overexpressed in 75-90% of human breast carcinomas. Kufe et al. (1984) *Hybridoma* 3: 223-232. For reviews, see Hilkens (1988) *Cancer Rev.* 11-12: 25-54; and Taylor-Papadimitriou, et al. (1990) *J. Nucl. Med. Allied Sci.* 34: 144-150. Mucin protein expression correlates with the degree
25 of breast tumor differentiation. Lundy et al. (1985) *Breast Cancer Res. Treat.* 5: 269-276. This overexpression appears to be controlled at the transcriptional level.

Overexpression of the *MUC1* gene in human breast carcinoma cells MCF-7 and ZR-75-1 appears to be regulated at the transcriptional level. Kufe et al. (1984); Kovarik
30 (1993) *J. Biol. Chem.* 268:9917-9926; and Abe et al. (1990) *J. Cell. Physiol.* 143: 226-231. The regulatory sequences of the *MUC1* gene have been cloned, including the approximately 0.9 kb upstream of the transcription start site which contains a TRE that appears to be involved in cell-specific transcription, depicted in SEQ ID NO:8. Abe et al.

(1993) *Proc. Natl. Acad. Sci. USA* 90: 282-286; Kovarik et al. (1993); and Kovarik et al. (1996) *J. Biol. Chem.* 271:18140-18147.

Any *MUC1*-TREs used in the present invention are derived from mammalian cells, including but not limited to, human cells. Preferably, the *MUC1*-TRE is human. In one embodiment, the *MUC1*-TRE may contain the entire 0.9 kb 5' flanking sequence of the *MUC1* gene. In other embodiments, the *MUC1*-TREs comprise the following sequences (relative to the transcription start site of the *MUC1* gene): about nt -725 to about nt +31, nt -743 to about nt +33, nt -750 to about nt +33, and nt -598 to about nt +485 (operably-linked to a promoter).

The *c-erbB2/neu* gene (*HER-2/neu* or *HER*) is a transforming gene that encodes a 185 kD epidermal growth factor receptor-related transmembrane glycoprotein. In humans, the *c-erbB2/neu* protein is expressed during fetal development, however, in adults, the protein is weakly detectable (by immunohistochemistry) in the epithelium of many normal tissues. Amplification and/or over-expression of the *c-erbB2/neu* gene has been associated with many human cancers, including breast, ovarian, uterine, prostate, stomach and lung cancers. The clinical consequences of the *c-erbB2/neu* protein over-expression have been best studied in breast and ovarian cancer. *c-erbB2/neu* protein over-expression occurs in 20 to 40% of intraductal carcinomas of the breast and 30% of ovarian cancers, and is associated with a poor prognosis in subcategories of both diseases. Human, rat and mouse *c-erbB2/neu* TREs have been identified and shown to confer *c-erbB2/neu* expressing cell specific activity. Tal et al. (1987) *Mol. Cell. Biol.* 7:2597-2601; Hudson et al. (1990) *J. Biol. Chem.* 265:4389-4393; Grooteclaes et al. (1994) *Cancer Res.* 54:4193-4199; Ishii et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:4374-4378; Scott et al. (1994) *J. Biol. Chem.* 269:19848-19858.

Thus, TREs derived from breast cell-specific TREs, including, but not limited to, those described herein, may be used in the present invention to generate stable adenovirus vectors that preferentially replicate in cells in which the TREs are functional, particularly cells derived from breast neoplasia. In one embodiment, the invention includes adenovirus vectors wherein the heterologous TREs are breast cell-specific. Accordingly, and by way of example, the invention includes adenovirus vectors in which the first heterologous TRE is a CEA-TRE and the second heterologous TRE is a *MUC1*-TRE, in which the first heterologous TRE is a *MUC1*-TRE and the second heterologous TRE is a CEA-TRE, in which the first heterologous TRE is a *MUC1*-TRE and the second heterologous TRE is a

HER-TRE, in which the first heterologous TRE is a *HER*-TRE and the second heterologous TRE is an *MUC1*-TRE, in which the first heterologous TRE is a *MUC1*-TRE and the second heterologous TRE is a *uPA*-TRE, in which the first heterologous TRE is a *uPA*-TRE and the second heterologous TRE is a *MUC1*-TRE, in which the first heterologous TRE is a *uPA*-TRE and the second heterologous TRE is an *HER*-TRE, in which the first heterologous TRE is an *HER*-TRE and the second heterologous TRE is a *uPA*-TRE, as described above.

Thus, TREs derived from colon cell-specific TREs, including, but not limited to, those described herein, may be used in the present invention to generate stable adenovirus vectors that preferentially replicate in cells in which the TREs are functional, particularly cells derived from colon neoplasia. In one embodiment, the invention includes adenovirus vectors wherein the heterologous TREs are colon cell-specific. Accordingly, and by way of example, the invention includes adenovirus vectors in which the first heterologous TRE is a *CEA*-TRE and the second heterologous TRE is a *uPA*-TRE, in which the first heterologous TRE is a *uPA*-TRE and the second heterologous TRE is a *CEA*-TRE, as described above.

As described above, some of the exemplary TREs are specific for more than one cell-type and thus, more than one type of neoplasia. Accordingly, adenovirus vectors of the present invention, as exemplified above, may be useful in the treatment of more than one type of neoplasm, as can be determined by the information provided herein.

The TREs listed above are provided as non-limiting examples of TREs that would function in the instant invention. Additional cell-specific TREs are known in the art, as are methods to identify and test cell specificity of suspected TREs. Further, and as noted above, the invention does not require that the TREs be derived from different genes. As long as the TRE sequences are sufficiently different, and the requisite functionality is displayed, the different TREs may be derived from the same gene.

For example, activity of a TRE can be determined as follows. A TRE polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested can be inserted into a vector containing a promoter (if no promoter element is present in the TRE) and an appropriate reporter gene encoding a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT), β -galactosidase (encoded by the *lacZ* gene), luciferase (encoded by the *luc* gene), alkaline

phosphatase, green fluorescent protein, and horse radish peroxidase. Such vectors and assays are readily available, from, inter alia, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE dextran.

After introduction of the TRE-reporter gene construct into a host cell under appropriate conditions, TRE activity may be measured by detection and/or quantitation of reporter gene-derived mRNA or protein product. The reporter gene protein can be detected directly (e.g., immunochemically) or through its enzymatic activity, if any, with an appropriate substrate. Generally, to determine cell specific activity of a TRE, the TRE-reporter gene constructs are introduced into a variety of cell types. The amount of TRE activity is determined in each cell type and compared to that of a reporter gene construct without the TRE. A TRE is cell specific when it is preferentially functional in a specific type of cell over a different type of cell.

For example, the specificity of *PB*-TRE activity for prostate cell that express the androgen receptor (AR) was demonstrated as follows. The region of the PB 5'-flanking DNA (nt -426 to nt +28) (SEQ ID NO:4) including the endogenous promoter sequences was inserted upstream of the firefly luciferase gene to generate a chimeric *PB*-TRE-luc plasmid. Cationic-mediated, transient transfection of LNCaP (PSA-producing and AR-producing prostate carcinoma cells) and PC-3 (PSA-deficient and AR-deficient prostate carcinoma cells) cells was performed. The results showed that LNCaP cells transfected with *PB*-TRE-luc had approximately 400 times more activity than untransfected cells, indicating that the *PB*-TRE was intact. Further, the overall luciferase activity recovered in the cellular extracts of transfected LNCaP cells was about 30-40-fold higher than that measured in the cellular extracts of transfected PC-3 cells. Thus, the results indicate that *PB*-TRE expression is preferentially functional in PSA-producing, AR-producing prostate carcinoma cells as compared to PSA-deficient, AR-deficient prostate carcinoma cells and that *PB*-TRE is capable of mediating specific expression in cells producing the androgen receptor.

(b) Exemplary genes under transcriptional control of the heterologous TREs

Any of the various serotypes of adenovirus can be used, such as Ad2, Ad5, Ad12, and Ad40. For purposes of illustration the serotype adenovirus 5 (Ad5) is exemplified herein.

In some embodiments, a cell specific, heterologous TRE is used to control transcription of an adenovirus gene and a second heterologous TRE, different from (i.e., not the same as) the first heterologous TRE, is used to control transcription of a second gene to provide an adenovirus vector so that replication-competence is preferentially achievable in target cells which allow for function of the cell-specific TRE. In addition, the two heterologous TREs are functional in the target cell. Preferably, the first adenovirus gene is essential for adenoviral replication, even more preferably, both genes are essential for adenoviral replication. Preferably, at least one of the genes is an early gene, such as E1A, E1B, E2, or E4. (E3 is not essential for viral replication.) Preferably, both genes under control of the heterologous TREs are early genes. More preferably, the early gene under cell-specific TRE control is E1A and/or E1B. Examples 1, 2 and 5 provide a more detailed description of such constructs.

The E1A gene is expressed immediately after viral infection (0-2 hours) and before any other viral genes. E1A protein acts as a *trans*-acting positive-acting transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2, E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are expressed during early times after Ad5 infection. Flint (1982) *Biochem. Biophys. Acta* 651:175-208; Flint (1986) *Advances Virus Research* 31:169-228; Grand (1987) *Biochem. J.* 241:25-38. In the absence of a functional E1A gene, viral infection does not proceed, because the gene products necessary for viral DNA replication are not produced. Nevins (1989) *Adv. Virus Res.* 31:35-81. The transcription start site of Ad5 E1A is at nt 498 and the ATG start site of the E1A protein is at nt 560 in the virus genome.

The E1B protein functions *in trans* and is necessary for transport of late mRNA from the nucleus to the cytoplasm. Defects in E1B expression result in poor expression of late viral proteins and an inability to shut off host cell protein synthesis. The promoter of E1B has been implicated as the defining element of difference in the host range of Ad40 and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis. Bailey et al. (1993) *Virology* 193:631; Bailey et al. (1994) *Virology* 202:695-706. The

E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box.

Accordingly, in one embodiment, the adenovirus E1A gene is under transcriptional control of the cell specific, heterologous TRE. In another embodiment, the adenovirus E1B gene is under transcriptional control of the cell specific, heterologous TRE. In another embodiment, both the adenovirus E1A and E1B genes are under transcriptional control of two different heterologous TREs, preferably both TREs are cell specific.

The E2 region of adenovirus codes for proteins related to replication of the adenoviral genome, including the 72 kD DNA-binding protein, the 80 kD precursor terminal protein and the viral DNA polymerase. The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units, respectively. While the E2 late promoter is transiently active during late stages of infection and is independent of the E1A transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site. For a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Micro. and Imm.* (1995) 199 part 3:177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33 kD protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a heterologous TRE having SpeI ends into the SpeI site in the plus strand would disrupt the endogenous E2 early promoter of Ad5 and should allow TRE-regulated expression of E2 transcripts.

The E4 gene has a number of transcription products. The E4 region codes for two polypeptides which are responsible for stimulating the replication of viral genomic DNA and for stimulating late gene expression. The protein products of open reading frames

(ORFs) 3 and 6 can both perform these functions by binding the 55-kD protein from E1B and heterodimers of E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55-kD protein for activity while the ORF 3 protein does not. In the absence of functional protein from ORF 3 and ORF 6, plaques are produced with an efficiency less than 10^{-6} that of wild type virus. To restrict further the viral replication to target cells, E4 ORFs 1-3 can be deleted, making viral DNA replication and late gene synthesis dependent on E4 ORF 6 protein. By combining such a mutant with sequences in which the E1B region is regulated by a target cell-specific TRE, a virus can be obtained in which both the E1B function and E4 function are dependent on the target cell-specific TRE driving E1B.

The major late genes relevant to the subject invention are L1, L2 and L3, which encode proteins of the Ad5 virus virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. The late genes are all under the control of the major late promoter (MLP), which is located in Ad5 at +5986 to +6048.

In one embodiment, the adenovirus E4 gene is under transcriptional control of the cell specific, heterologous TRE. In another embodiment, an adenovirus late gene is under transcriptional control of the cell specific, heterologous TRE. In another embodiment, one early gene and one late gene are under transcriptional control of different heterologous TREs.

In addition to conferring selective cytotoxic and/or cytolytic activity by virtue of preferential replication competence in cells which allow function of the heterologous TREs, the adenovirus vectors of this invention can further include a heterologous polynucleotide (transgene) under the control of a heterologous TRE. In this way, various genetic capabilities may be introduced into target cells. For example, in certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of the target cell. This could be accomplished by coupling the cell-specific replicative cytotoxic activity with cell-specific expression of, for example, HSV-tk and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these types of transgenes may also confer a bystander effect.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding cytotoxic proteins, such as the A chains of diphtheria toxin, ricin or abrin (Palmiter et al. (1987) *Cell* 50: 435; Maxwell et al. (1987) *Mol. Cell. Biol.* 7:

1576; Behringer et al. (1988) *Genes Dev.* 2: 453; Messing et al. (1992) *Neuron* 8: 507; Piatak et al. (1988) *J. Biol. Chem.* 263: 4937; Lamb et al. (1985) *Eur. J. Biochem.* 148: 265; Frankel et al. (1989) *Mol. Cell. Biol.* 9: 415), genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among
5 other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; viral or other pathogenic proteins, where the pathogen proliferates intracellularly, genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. aprotinin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of
10 interest include potential therapeutic genes such as cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN- α , - β , - γ , TNF- α , - β , TGF- α , - β , NGF, and the like. The positive effector genes could be used in an early phase, followed by cytotoxic activity due to replication.

In some embodiments, the adenovirus death protein (ADP), encoded within the E3
15 region, is maintained (i.e., contained) in the adenovirus vector. The ADP gene, under control of the major late promoter (MLP), appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson et al. (1996) *J. Virol.* 70(4):2296; Tollefson et al. (1992) *J. Virol.* 66(6):3633. Thus, adenoviral vectors containing the ADP gene may render the adenoviral vector more potent, making possible more effective
20 treatment and/or a lower dosage requirement.

Accordingly, the invention provides adenovirus vectors in which a first adenovirus gene is in under transcriptional control of a first heterologous cell-specific TRE and a polynucleotide sequence encoding an ADP under control of a second heterologous TRE, which is different from the first TRE but functional in the same cell as the first TRE,
25 preferably the first adenovirus gene is essential for replication. A DNA sequence encoding an ADP and the amino acid sequence of an ADP are depicted in SEQ ID NO:9 and SEQ ID NO:10, respectively. Briefly, an ADP coding sequence is obtained preferably from Ad2 (since this is the strain in which ADP has been more fully characterized) using techniques known in the art, such as PCR. Preferably, the Y leader (which is an important
30 sequence for correct expression of late genes) is also obtained and ligated to the ADP coding sequence. The ADP coding sequence (with or without the Y leader) can then be introduced into the adenoviral genome, for example, in the E3 region (where the ADP coding sequence will be driven by the MLP). The ADP coding sequence could also be

inserted in other locations of the adenovirus genome, such as the E4 region. Alternatively, the ADP coding sequence could be operably linked to a heterologous TRE, including, but not limited to, another viral TRE, a tissue specific TRE such as that of *AFP*, *CEA*, *hKLK2*, *MUC1*, *PSE* and *PB*.

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It is understood that the present invention does not exclude adenovirus vectors containing additional genes under control of the heterologous TREs. Accordingly, the invention provides adenoviral vectors comprising a third gene under transcriptional control of a third heterologous TRE where all of the heterologous TREs are different from each other in polynucleotide sequence but all are functional in the same cell. Preferably, the third gene is one that contributes to cytotoxicity (whether direct and/or indirect), more preferably one that contributes to and/or enhances cell death, and even more preferably the third gene is essential from adenovirus replication. Preferably the third heterologous TRE is target cell specific. For example, an adenovirus vector may contain a *PB*-TRE, a *PSE*-TRE and an *hKLK2*-TRE, each prostate cell specific and each controlling the transcription of a different gene.

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As is known in the art and described herein, the ability of enhancers to increase transcription of an operably linked gene is independent of its orientation and distance relative to the gene. Accordingly, the invention provides adenoviral vectors comprising at least an additional gene (beyond the first and the second genes) under transcriptional control of the second heterologous TRE. Preferably, the additional gene is one that contributes to cytotoxicity (whether direct and/or indirect), more preferably one that enhances cell death, and even more preferably the third gene is essential from adenovirus replication.

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(c) Delivery of adenoviral vectors to cells

The adenoviral vectors can be used in a variety of forms, including, but not limited to, naked polynucleotide (usually DNA) constructs; polynucleotide constructs complexed with agents to facilitate entry into cells, such as cationic liposomes or other compounds such as polylysine; packaged into infectious adenovirus particles (which may render the adenoviral vector(s) more immunogenic); packaged into other particulate viral forms such as HSV or AAV; complexed with agents to enhance or dampen an immune response;

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complexed with agents that facilitate *in vivo* transfection, such as DOTMATM, DOTAPTM, and polyamines.

If an adenoviral vector is packaged into an adenovirus, the adenovirus itself may be selected to further enhance targeting. For example, adenovirus fibers mediate primary contact with cellular receptor(s) aiding in tropism. See, e.g., Amberg et al. (1997) *Virol.* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type and/or reduced affinity for non-target cell types, such subgenus (or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art (such as calcium phosphate precipitation and electroporation), and direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

If used in a packaged adenovirus, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about 10^4 to about 10^{14} . The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 μ g to 1000 μ g of an adenoviral vector can be administered. The adenoviral vectors may be administered one or more times, depending upon the intended use and the immune response potential of the host or may be administered as multiple simultaneous injections.

If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an amount to be administered is based on standard knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined empirically.

In some embodiments, an adenovirus vector(s) is complexed to a hydrophilic polymer to create a masked adenovirus. The hydrophilic polymer is attached (covalently or non-covalently) to the capsid proteins of the adenovirus, particularly the hexon and fiber proteins. In preferred embodiments, the adenovirus vectors of the instant invention are complexed with masking agents to create masked adenovirus vectors. Masked

adenoviruses are advantageous due to (a) the masking of the adenovirus surface to adenovirus neutralizing antibodies or opsinins which are in circulation, and (b) increasing systemic circulation time of adenovirus particles by reduction of non-specific clearance mechanism in the body (i.e., macrophages, etc.). In the *in vivo* context, the systemic delivery of a masked adenovirus results in a longer circulation of viral particles, less immunogenicity, and increased biodistribution with a decrease in clearance by the liver and spleen. Extensive research has been done on modification of proteins and lipids with hydrophilic polymers (especially PEG), but the inventors are unaware of any other use of masking agents in conjunction with adenovirus or adenovirus constructs.

Accordingly, the invention provides an adenovirus complexed with a masking agent. Preferably, the masking agent is PEG. A schematic of one method of making a masked adenovirus is depicted in Figure 13 (see also Example 7). A preferred embodiment is a masked adenovirus comprising an adenovirus vector(s) described herein, with a more preferred embodiment comprising a pegylated adenovirus comprising an adenovirus described herein. The invention also provides methods to make and use these masked adenoviruses, which are evident from the description herein.

The masking agent may be of various molecular weights, as long as the desired complex and requisite functionality is obtained. Most masking agents obtained from commercial sources are normally polydisperse in relation to the stated molecular weight (i.e., the masking agent is supplied in a distribution of molecular weights surrounding the nominal molecular weight). Masking agents useful in masking adenoviruses according to the instant invention may have nominal weights of about 2000 to about 50,000; preferably, about 2500 to about 30,000; preferably, about 3000 to about 25,000; more preferably, about 5000 to about 20,000. Preferably, the nominal molecular weight is less than about 20,000, more preferably less than about 10,000, more preferably less than about 7500, more preferably less than about 5000. Preferably, the masking agent is PEG with a nominal molecular weight of less than about 5000 Da. Mixtures of different weight masking agents are also contemplated.

The masking may be covalently or non-covalently attached. In the case of non-covalent attachment, the attachment may be via electrostatic, hydrophobic, or affinity interactions. The masking agents used for non-covalent attachment may be modified masking agents (i.e., the masking agent is synthesized or modified to contain particular chemical moieties not normally found in the masking agent). Masking agents useful for

electrostatic attachment to adenoviral vectors will be masking agents which contain, or have been modified or synthesized to contain, charged moieties which bind to the adenovirus surface by electrostatic interaction. Negatively charged masking agents include masking agents which contain phosphate groups, sulfate groups, carboxy groups, and the like. Quaternary amine groups are useful as positively charged moieties for electrostatic, non-covalent attachment of masking agents to adenovirus. Masking agents containing or modified or synthesized to contain hydrophobic groups, such as lipids (*e.g.*, phosphatidylethanolamine and the like) and other hydrophobic groups (such as phenyl groups or long alkyl chains), can be complexed to adenoviral vectors by hydrophobic interaction with stable hydrophobic regions on the virus. Affinity masking agents can be made using any small molecule, peptide or protein which binds to adenovirus. The affinity and hydrophobic moieties may be attached to the masking agent by any method known in the art, preferably by chemical crosslinking with a chemical crosslinker.

If the masking agent is covalently attached, a chemical crosslinker is preferably used to covalently bond the masking agent to the adenovirus. The crosslinker may be any crosslinker capable of creating a covalent linkage or bridge between the masking agent and the adenovirus. Direct crosslinking, in which the adenovirus, masking agent and a separate crosslinker molecule are reacted, may be employed to create covalently masked adenovirus, using any chemical crosslinker known in the art which will create crosslinks between the masking agent and protein. Either the masking agent or the adenovirus may be modified prior to the crosslinking reaction, so that the chemical crosslinker will react with the two molecules (*e.g.*, the masking agent may be modified to add amine groups, allowing it to be crosslinked to the adenovirus by crosslinking agents which react with amines).

Preferably, either the masking agent or the adenovirus is first activated by reaction with a crosslinking agent. Unreacted crosslinker is then removed from the masking agent or adenovirus. The activation reaction preferably results in one or two molecules of crosslinking agent per molecule of masking agent, more preferably a single molecule of crosslinking agent per molecule of masking agent. The activated masking agent or adenovirus is then mixed with adenovirus (if the masking agent is activated) or masking agent (if the adenovirus is activated) under the appropriate reaction conditions to form masked adenovirus. Preferably, the masking agent is activated, then reacted with adenovirus.

The preferred masking agent is PEG. Preferred activated PEGs include, but are not limited to: nucleophilic crosslinking PEGs such as end terminal amine PEG, PEG amino acid esters, PEG hydrazine hydrochloride, thiol PEGs, and the like; carboxyl PEGs including succinate PEG, carboxymethylated PEG, PEG-propionic acid, and PEG amino acids; 5 sulfhydryl-selective PEGs such as PEG-maleimide, PEG-orthopyridyl-disulfide and the like; heterofunctional PEGs including amines and acids PEG, NHS-maleimide PEG and NHS-vinylsulfone PEG; PEG silanes; biotin PEGs; Vinyl derivatives of PEG such as allyl PEG, PEG acrylate, PEG methacrylate, and the like; and electrophilic active PEGs, including PEG succinimidyl succinate, PEG succinimidyl succinamide, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, PEG2-NHS, succinimidyl 10 esters of amino acid PEGs, pendant modified PEG NHS esters (such as those available from Innophase, Inc.), PEG-glycidyl ether (epoxide), PEG-oxycarbonylimidazole, PEG nitrophenyl carbonates, PEG trichlorophenyl carbonates, PEG treslate, PEG-aldehyde, PEG-isocyanate, copolymers of PEG allyl ether and maleic anhydride, PEG vinylsulfone, 15 and other activated PEGs as will be apparent to one of skill in the art. The activated PEG is preferably PEG-N-hydroxysuccinimidyl succinamide or PEG-succinimidyl succinate, more preferably PEG-N-hydroxysuccinimidyl succinamide.

Host Cells

20 The present invention also provides host cells comprising (i.e., transformed with) the adenoviral vectors described herein. Both prokaryotic and eukaryotic host cells can be used as long as sequence requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Prokaryotic host include bacterial cells, for example, *E. coli* and mycobacteria. Among 25 eukaryotic host cells are yeast, insect, avian, amphibian, plant and mammalian host cells. Host systems are known in the art and need not be described in detail herein. Suitable host cells also include any cells that produce proteins and other factors necessary for expression of the gene under control of the heterologous TREs, such factors necessary for said expression are produced naturally or recombinantly.

30 Suitable host cells for the adenovirus include any eukaryotic cell type that allows function of the heterologous TREs, preferably mammalian. For example, if the heterologous TRE(s) used is prostate cell-specific, the cells are preferably prostate cells, for example LNCaP cells. The prostate cells used may or may not be producing an

androgen receptor, depending on whether the promoter used is androgen-inducible. If an androgen-inducible promoter is used, non-androgen receptor producing cells, such as HLF, HLE, and 3T3 and the non-AR-producing prostate cancer cells PC3 and DU145 can be used, provided an androgen receptor-encoding expression vector is introduced into the cells along with the adenovirus. If the heterologous TRE(s) used is derived from the AFP gene, for example, suitable host cells include any cell type that produces AFP, including but not limited to, Hep3B, HepG2, HuH7, HuH1/C12. Activity of a given TRE in a given cell can be assessed by measuring the level of expression of a operably-linked reporter gene using standard assays. The comparison of expression between cells in which the TRE is suspected of being functional and the control cell indicates the presence or absence of transcriptional enhancement.

Comparisons between or among various TREs can be assessed by measuring and comparing levels of expression within a single cell line. It is understood that absolute transcriptional activity of a TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of a TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the CMV immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

Compositions

The present invention also includes compositions, including pharmaceutical compositions, containing the adenoviral vectors described herein. Such compositions are useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an individual. Preferably, these compositions further comprise a pharmaceutically acceptable excipient. These compositions, which can comprise an effective amount of an adenoviral vector of this invention in a pharmaceutically acceptable excipient, are suitable for systemic administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions

and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing (1990). Compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the invention.

Kits

The present invention also encompasses kits containing an adenoviral vector of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits embodied by this invention allow someone to detect the presence of target cells in a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

Preparation of the adenovirus vectors of the invention

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Generally, heterologous TREs are inserted 5' to the adenoviral genes of interest, preferably one or more early genes (although late gene(s) may be used). Heterologous TREs can be prepared using oligonucleotide synthesis (if the sequence is known) or recombinant methods (such as PCR and/or restriction enzymes). Convenient restriction sites, either in the natural adeno-DNA sequence or introduced by methods such as PCR or site-directed mutagenesis, provide an insertion site for the heterologous TREs. Accordingly, convenient restriction sites for annealing (i.e., inserting) heterologous TREs can be engineered onto the 5' and 3' ends of the heterologous TRE using standard recombinant methods, such as PCR.

Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art such as chemical synthesis recombinant methods and/or obtained from biological sources.

Adenovirus vectors containing all replication-essential elements, with the desired elements (e.g., E1A) under control of heterologous TREs, are conveniently prepared by homologous recombination or *in vitro* ligation of two plasmids, one providing the left-hand portion of adenovirus and the other providing the right-hand portion. The resultant
5 adenovirus vector contains at least two different heterologous TREs, with at least one of the heterologous TREs cell-specific and one adenovirus gene under control of a first heterologous TRE and a second gene under control of a second heterologous TRE. If homologous recombination is used, the two plasmids should share at least about 500 bp of sequence overlap. Each plasmid, as desired, may be independently manipulated, followed
10 by cotransfection in a competent host, providing complementing genes as appropriate, or the appropriate transcription factors for initiation of transcription from the heterologous TREs for propagation of the adenovirus. Plasmids are generally introduced into a suitable host cell such as 293 cells using appropriate means of transduction, such as cationic liposomes. Alternatively, *in vitro* ligation of the right and left-hand portions of the
15 adenovirus genome can be used to construct recombinant adenovirus derivative containing all the replication-essential portions of adenovirus genome. Berkner et al. (1983) *Nucleic Acid Research* 11:6003–6020; Bridge et al. (1989) *J. Virol.* 63:631–638.

For convenience, plasmids are available that provide the necessary portions of adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type
20 left-hand end of Ad5, from Adenovirus 5 nt 22 to 5790. pBHG10 (Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806; Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3. The deletion in E3 provides room in the virus to insert a 3-kb of TRE sequence or transgene sequence without deleting the endogenous enhancer-promoter. The gene for E3 is located on the opposite strand from E4
25 (r-strand). pBHG11 [Bett et al. (1994)] provides an even larger E3 deletion (an additional 0.3 kb is deleted).

For manipulation of the early genes, the transcription start site of Ad5 E1A is at nt 498 and the ATG start site of this gene's coding segment is at nt 560 in the virus genome. This region can be used for insertion of a heterologous TRE. A restriction site may be
30 introduced by employing PCR, where the primer that is employed may be limited to the Ad5 genome, or may involve a portion of the plasmid carrying the Ad5 genomic DNA. For example, where pBR322 is used, the primers may use the EcoRI site in the pBR322 backbone and the XbaI site at nt 1339 of Ad5. By carrying out the PCR in two steps,

where overlapping primers at the center of the region introduce a 30 sequence change resulting in a unique restriction site, one can provide for insertion of a heterologous TRE at that site.

5 A similar strategy may be used for insertion of a heterologous TRE to regulate E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box. This region extends from Ad5 nt 1636 to 1701. By insertion of a cell-specific, heterologous TRE in this region, one can provide for cell-specific transcription of the E1B gene. By employing the left-hand region modified with a cell-specific TRE regulating E1A as the template for introducing a second, different cell-specific TRE to
10 regulate E1B, the resulting adenovirus vector will be dependent upon the cell-specific transcription factors for expression of both E1A and E1B. Examples 1, 2 and 5 provide a more detailed description of how such constructs can be prepared.

Similarly, a heterologous TRE may be inserted upstream of the E2 gene to make its expression cell-specific. The E2 early promoter, mapping in Ad5 from 27050-27150,
15 consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site (for a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Microbiol. and Immunol.* (1995) 199 part 3:177-194).

20 The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable to genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33-kD protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates
25 the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a heterologous TRE having SpeI ends into the SpeI site in the plus-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow TRE regulated expression of E2 transcripts.

30 For E4, one must use the right hand portion of the adenovirus genome. The E4 transcription start site is predominantly at nt 35609, the TATA box at nt 35638 and the first AUG/CUG of ORF1 is at nt 35532. Virtanen et al. (1984) *J. Virol.* 51: 822-831. Using any of the above strategies for the other genes, a heterologous TRE may be

introduced upstream from the transcription start site. For the construction of mutants in the E4 region, the co-transfection and homologous recombination are performed in W162 cells (Weinberg *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:5383–5386) which provide E4 proteins *in trans* to complement defects in synthesis of these proteins.

5 Methods of packaging adenovirus polynucleotides into adenovirus particles are known in the art and are described in the Examples.

 The methods of preparation of masked adenovirus vary according to masking agent and the mode of attachment (covalent or non-covalent). Masked adenovirus with non-covalently attached masking agent is prepared by thoroughly and intimately mixing the
10 adenovirus and the masking agent. Covalent crosslinking is achieved by mixing the reaction components under conditions appropriate to the crosslinking reagent. Chemical crosslinking protocols are well known in the art. The exact reaction conditions for any given chemical crosslinker will vary according to the chemistry of the crosslinker and the modifications (if any) to the PEG and the adenovirus, as will be apparent to one of skill in
15 the art. When the masking agent is PEG, the molar ratio of adenovirus to PEG is preferably between $1:1 \times 10^6$ and $1:1 \times 10^7$, more preferably about $1:4 \times 10^6$. For the preferred activated PEG, PEG-NHS-succinamide, the activated PEG is preferably between 0.5 to 5 mM in the crosslinking reaction, more preferably about 2 mM. Preferably adenovirus in the crosslinking reaction is between 10^6 and 10^{12} , more preferably about $5 \times$
20 10^9 . Using the preferred activated PEG, the pH of the crosslinking reaction is preferably between about 7 and 9, more preferably between about 7.5 and 8, and the reaction is preferably run for 10 to 30 minutes at a temperature ranging from about 4° C to room temperature (about 20° C).

 Following the crosslinking reaction, the masked adenovirus is separated from the
25 reaction components. The separation may be accomplished by any method known to one of skill in the art, including chromatographic methods such as size exclusion chromatography, ion exchange chromatography or hydrophobic interaction chromatography, electrophoretic methods, or filtration methods such as dialysis, diafiltration or ultrafiltration.

30 Methods using the adenovirus vectors of the invention

 The subject vectors can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods

using the adenoviral vectors described above. In one embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a cell, preferably a eukaryotic cell, more preferably a mammalian cell.

5 In one embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a target cell, preferably a neoplastic cell. In another embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a prostate cell. In another embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a liver cell. In another embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a breast cancer
10 cell. In another embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a colon cancer cell.

In one embodiment, methods are provided for conferring selective cytotoxicity in cells which allow function of the target cell-specific TRE, comprising contacting cells with an adenovirus vector described herein, such that the adenovirus vector(s) enters, i.e.,
15 transduces the cell(s). Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, ³H-thymidine incorporation, and/or lysis. See Example 3, Fig. 3.

In another embodiment, methods are provided for propagating an adenovirus specific for cells which allow function of the heterologous TREs, preferably eukaryotic cells, more preferably mammalian cells. These methods entail combining an adenovirus
20 vector with mammalian cells which allow function of the heterologous TREs, whereby said adenovirus is propagated.

Another embodiment provides methods of killing cells that allow a heterologous TRE to function comprising combining the mixture of cells with an adenovirus vector of the present invention. The mixture of cells is generally a mixture of cancerous cells in
25 which the heterologous TREs are functional and normal cells, and can be an *in vivo* mixture or *in vitro* mixture.

The invention also includes methods for detecting cells in which the heterologous TREs are functional in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal),
30 whether in an experimental or clinical setting. For these methods, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. A suitable biological sample is one in which target cells may be or are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which target

cancerous cells are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to promote assay conditions such as selective enrichment and/or solubilization. In these methods, target cells can be detected using *in vitro* assays that detect proliferation, which are
5 standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yields) and plaque assays (which measure infectious particles per cell). Also, propagation can be detected by measuring specific adenoviral DNA replication, which are also standard assays.

The invention also provides methods of modifying the genotype of a target cell,
10 comprising contacting the target cell with an adenovirus vector described herein, wherein the adenoviral vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor
15 cell. As used herein, "tumor cells" and "tumor" refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ³H-thymidine incorporation assay, or
20 counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage. "Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein. See Example 4, Fig.6.

The invention also provides methods of lowering the levels of a tumor cell marker in an individual, comprising administering to the individual an adenoviral vector of the present invention, wherein the adenoviral vector is selectively cytotoxic toward cells
25 producing the tumor cell marker. Tumor cell markers include, but are not limited to, PSA, carcinoembryonic antigen and hK2. Methods of measuring the levels of a tumor cell marker are known to those of ordinary skill in the art and include, but are not limited to,
30 immunological assays, such as enzyme-linked immunosorbent assay (ELISA), using antibodies specific for the tumor cell marker. In general, a biological sample is obtained

from the individual to be tested, and a suitable assay, such as an ELISA, is performed on the biological sample. See Example 4, Fig. 7.

The invention also provides methods of treatment, in which an effective amount of an adenoviral vector(s) described herein is administered to an individual. For example, treatment using an adenoviral vector(s) in which at least one heterologous TRE is specific for prostate cells (e.g., *PSE*-TRE, *PB*-TRE, and/or *hKLK2*-TRE) is indicated in individuals with prostate-associated diseases as described above, such as hyperplasia and cancer. In this example, also indicated are individuals who are considered to be at risk for developing prostate-associated diseases, such as those who have had disease which has been resected and those who have had a family history of prostate-associated diseases. Determination of suitability of administering adenoviral vector(s) of the invention will depend, inter alia, on assessable clinical parameters such as serological indications and histological examination of tissue biopsies. Generally, a pharmaceutical composition comprising an adenoviral vector(s) is administered. Pharmaceutical compositions are described above.

The amount of adenoviral vector(s) to be administered will depend on several factors, such as route of administration, the condition of the individual, the degree of aggressiveness of the disease, the particular heterologous TREs employed, and the particular vector construct (i.e., which adenovirus genes are under heterologous TRE control).

If administered as a packaged adenovirus, from about 10^4 to about 10^{14} , preferably from about 10^4 to about 10^{12} , more preferably from about 10^4 to about 10^{10} . If administered as a polynucleotide construct (i.e., not packaged as a virus), about 0.01 μg to about 100 μg can be administered, preferably 0.1 μg to about 500 μg , more preferably about 0.5 μg to about 200 μg . More than one adenoviral vector can be administered, either simultaneously or sequentially. Administrations are typically given periodically, while monitoring any response. Administration can be given, for example, intratumorally, intravenously or intraperitoneally.

The adenoviral vectors of the invention can be used alone or in conjunction with other active agents, such as chemotherapeutics, that promote the desired objective.

The following examples are provided to illustrate but not limit the invention.

EXAMPLES

EXAMPLE 1

Generation of adenovirus vector constructs in which a first adenovirus gene is under transcriptional control of an hKLK2-TRE and a second adenovirus gene is under transcriptional control of a PSE-TRE

5 *1.1 Construction of adenovirus constructs in which expression of one adenovirus gene is controlled by an hKLK2-TRE*

To generate hKLK2-TRE adenovirus constructs, four hKLK2-TRE fragments which contain at least the hKLK2 minimal promoter were amplified using the DNA sequence from approximately 12,000 bp lying upstream of the first exon in hKLK2 (SEQ ID NO:3) and synthetic oligonucleotides as described below. The four constructs were generated by
10 ligating these fragments into pGEM-T vector.

- CN294 is a pGEM-T vector derivative containing an hKLK2 full promoter. The fragment was amplified by PCR with oligonucleotide 42.100.1: 5'-GAT CAC CGG TGT CCA CGG CCA GGT GGT GC-3' (SEQ ID NO:11) (PinAI site underlined),
15 which is complementary to the 5'-untranslated region (UTR) of the first exon in hKLK2, in combination with 42.100.2 (5'-GAT CAC CGG TGC TCA CGC CTG TAA TCT CAT CAC-3'; SEQ ID NO:12; PinAI site underlined). 42.100.2 corresponds to the upstream region of the hKLK2 promoter.

- CN296 is a pGEM-T vector derivative containing an hKLK2 fragment from nt -2247 to nt +33, which was amplified by PCR with oligonucleotides 42.100.1 and 42.100.3 (5'-GAT CAC CGG TGG TTT GGG ATG GCA TGG CTT TGG-3'; SEQ ID NO:13, PinAI site underlined). 42.100.3 corresponds to a region approximately 2300 bp upstream of hKLK2.

- CN317 is a pGEM-T derivative containing the hKLK2 minimal promoter. A PCR fragment corresponding to the hKLK2 5'-UTR from nt -323 to nt +33 was amplified with two synthetic oligonucleotides; 42.100.1 and 43.121.1 (5'-GAT CAC CGG TAA AGA ATC AGT GAT CAT CCC AAC-3'; SEQ ID NO:14, PinAI site underlined).

- CN310 is a pGEM-T vector derivative containing an hKLK2 full promoter and is identical to CN294 except EagI sites flank the insert. The fragment was amplified by PCR with oligonucleotide 42.174.1 (5'-GAT CCG GCC GTG GTG CTC ACG CCT GTA ATC-3'; SEQ ID NO:15, EagI site underlined) in combination with 42.174.2 (5'-

GAT CCG GCC GTG TCC ACG GCC AGG TGG TGC AG-3'; SEQ ID NO:16; EagI site underlined).

hKLK2 promoter-driven E1A Ad5 plasmid CN303

5 CN303 was produced by inserting the *hKLK2* promoter just upstream of the E1A coding segment in a derivative of pXC-1, a plasmid containing the left hand end of the Ad5 genome, as follows.

- CN124 is a derivative of construct pXC-1 which contains the wild-type left hand end of Ad5, including both E1A and E1B (McKinnon (1982) *Gene* 19:33-42). CN124
10 also has, among other alterations, an artificial PinAI site at Ad5 nt 547 (just upstream of the E1A transcriptional start at nt 560 and the E1A coding segment beginning with ATG at 610). CN124 was linearized with PinAI and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs).
- CN294 was digested with PinAI to free the *hKLK2* promoter. The *hKLK2* promoter
15 was then ligated into the PinAI linearized CN124, producing CN303. CN304 is similar to CN303 except for the *hKLK2* promoter fragment is in the reverse orientation.

20 Thus, construct CN303 contains the *hKLK2* promoter inserted upstream of and operably linked to the E1A coding segment in the Adenovirus 5 genome.

hKLK2 promoter-driven E1B Ad5 plasmid CN316

CN316 was produced by inserting the *hKLK2*-promoter just upstream of the E1B coding segment in a derivative of pXC-1, a plasmid containing the left hand end of the
25 Ad5 genome, as follows.

- CN124, described above, also contains an artificial EagI site at Ad5 nt 1682, just
upstream of the E1B coding segment. The *hKLK2* promoter was excised from CN310
with EagI and inserted into CN124 digested with EagI to produce CN316. CN316
contains the *hKLK2* promoter immediately upstream of and operably linked to the E1B
30 coding segment.

1.2 Construction of adenovirus constructs in which expression of one adenovirus replication gene is controlled by an hKLK2-TRE and expression of another adenovirus replication gene is controlled by a PSE-TRE

5 *Ad5 construct comprising an hKLK2-TRE driven E1A and a PSE-TRE driven E1B (CN301)*

CN301 was generated from CN125 by inserting an *hKLK2* promoter upstream of the E1A gene as follows.

- 10 • CN125 is a pXC-1 derivative in which expression of the E1B gene is driven by *PSE*. A PinAI site lies upstream of the E1A gene, whose expression is driven by its wild-type promoter. CN125 was created by inserting *PSE* as an *EagI* fragment from construct CN105 into the *EagI* site immediately upstream of the E1B gene in CN124. CN105 contains the *PSE* region from -5322 to -3875 relative to the *PSA* transcription start site.
- 15 • The *hKLK2*-TRE fragment was freed from CN294 by PinAI digestion and ligated into PinAI digested CN125 to create CN301.

The CN301 construct contains the *hKLK2* promoter immediately upstream of and operably-linked to the E1A gene and the *PSE*-TRE immediately upstream of and operably-linked to the E1B gene.

20

Ad5 constructs comprising PSE-TRE driven E1A gene and hKLK2 promoter driven E1B gene (CN323)

CN323 was constructed as follows so that the expression of E1A is mediated by *PSE*-TRE, and expression of E1B is mediated by an *hKLK2* promoter.

25

- CN314 is a plasmid containing a *PSE*-TRE fragment in pGEM-T vector. This *PSE* fragment was amplified from CN706, an adenoviral construct in which a *PSE*-TRE (SEQ ID NO:2) drives expression of the E1A transcription unit in Ad5, with two synthetic oligonucleotides:

30

51.10.1 (5'-CTC ATT TTC AGT CAC CGG TAA GCT TGG-3'; SEQ ID NO:17) and
51.10.2 (5'-GAG CCG CTC CGA CAC CGG TAC CTC-3'; SEQ ID NO:18).

- The *PSE*-TRE fragment was isolated by digesting CN314 with PinAI and ligated into PinAI digested CN316 (described above).

The CN323 construct is a plasmid containing *PSE*-TRE immediately upstream of and operably-linked to the E1A gene and the *hKLK2* promoter immediately upstream of and operably-linked to the E1B gene.

5 *1.3 Construction of additional adenoviral constructs comprising a first adenoviral gene under transcriptional control of an hKLK2-TRE and a second adenoviral gene under transcriptional control of a PSE-TREs*

- 10 • CN306 was derived from CN124 by removing the endogenous 64-nucleotide E1A promoter.
- 15 • CN421 was constructed by inserting an *hKLK2*-TRE (comprising an *hKLK2* enhancer from nucleotides -5155 to -3387 relative to the *hKLK2* gene transcription start site (nucleotides 6859 to 8627 of SEQ ID NO:3) and an *hKLK2* minimal promoter as in CN379 into CN306. The *hKLK2*-TRE fragment was amplified by PCR from CN379, digested with PinAI and ligated into similarly cut CN306, to produce CN421.
- 20 • CN438 was constructed by inserting an *hKLK2*-TRE (comprising an *hKLK2* enhancer from nucleotides -4814 to -3643 relative to the *hKLK2* gene transcription start site (nucleotides 7200 to 8371 of SEQ ID NO:3) and a minimal *hKLK2* promoter as in CN390 into CN306. The enhancer fragment was amplified by PCR from CN390, digested with PinAI and ligated into similarly cut CN306, to produce CN438.
- 25 • CN321 was created from CN306 by inserting a large *PSE*-TRE amplified from CN96 (see US Patent No. 5,698,443; Rodriguez et al. (1997)).
- 30 • CN416 was constructed by inserting an *hKLK2*-TRE (comprising an *hKLK2* enhancer from nucleotides -5155 to -3387 relative to the *hKLK2* gene transcription start site (nucleotides 6859 to 8627 of SEQ ID NO:3) and an *hKLK2* minimal promoter as in CN379 into CN321. The enhancer fragment was amplified by PCR from CN379, digested with EagI and ligated into similarly cut CN321, to generate CN416.
- 35 • CN422 was constructed by inserting an *hKLK2*-TRE (comprising an *hKLK2* enhancer from nucleotides -5155 to -3387 relative to the *hKLK2* gene transcription start site (nucleotides 6859 to 8627 of SEQ ID NO:3) and an *hKLK2* minimal promoter as in

CN379 into CN369. CN369 is a derivative of CN306 in which the endogenous E1B promoter was removed. The *hKLK2*-TRE was amplified from CN379, digested with *EagI*, and ligated into similarly cut CN369 to produce CN422.

- CN444 was constructed by replacing the *hKLK2*-TRE of CN442 with an *hKLK2*-TRE comprising an *hKLK2* enhancer from nucleotides -4814 to -3643 relative to the *hKLK2* transcription start site (nucleotides 7200 to 8371 of SEQ ID NO:3) and a minimal *hKLK2* promoter, as in CN390. The *hKLK2*-TRE was amplified from CN390, digested with *EagI*, and ligated into similarly cut CN369, to produce CN444.

- CN446 is similar to CN444, except that the endogenous E1B promoter was not removed. The *hKLK2*-TRE was amplified from CN390, digested with *EagI*, and ligated into similarly cut CN321, to produce CN446.

- CN459 and CN460 are similar to CN444, except that each contains an *hKLK2*-TRE comprising an *hKLK2* enhancer from nucleotides -3993 to -3643 relative to the *hKLK2* transcription start site and a *hKLK2* minimal promoter, as in CN396.

- CN463 was constructed by inserting an *hKLK2*-TRE (comprising an *hKLK2* enhancer from nucleotides -4814 to -3643 relative to the *hKLK2* transcription start site and a minimal *hKLK2* promoter, as in CN390, into CN251. The *hKLK2*-TRE was excised from CN446 with *EagI*, and ligated into similarly cut CN251, to produce CN463.

1.4 Generation of recombinant adenoviruses

Adenovirus containing *hKLK2*-TRE were generated by homologous recombination in 293 cells. Briefly, CN301 was co-transfected with BHG10 (which contains right hand end of the adenovirus genome), into 293 cells. The cells were overlaid with media, and infectious virus generated by *in vivo* recombination was detected by cytopathic effect and isolated. Plaque-purified stocks of an adenovirus vector, designated CN747, were established. The structure of the recombinant virus was characterized by PCR, restriction endonuclease digestion and Southern blot. CN747 is a full-length Ad5 with the *hKLK2* promoter driving the expression of E1A and a *PSE*-TRE driving expression of E1B.

Virus CN754 were generated with the same approach except that the CN301 plasmid was replaced with CN323. CN754 is a virus whose E1A and E1B are under the control of the *PSE*-TRE and the *hKLK2*-TRE, respectively.

Viruses CN753, CN755, CN759, CN761, CN763, CN764, CN765, CN767, CN768, CN769, CN770, CN772 and CN773 were generated using the method as described above, from the parent plasmids CN326, CN328, CN398, CN316, CN421, CN416, CN422, CN436, CN438, CN444, CN446, CN459, CN460, and CN463, respectively. These viral constructs are shown schematically in Figures 2A, 2B and 2B.

EXAMPLE 2

Construction of adenovirus vectors in which expression of one viral replication gene is controlled by a PSE-TRE and expression of another is controlled by a PB-TRE

2.1 Generation of adenovirus constructs in which expression of one adenovirus gene is controlled by a PB-TRE

The 454 nucleotide fragment (nt about -426 to about +28) of the rat *PB*-TRE, which contains two androgen response elements (ARE sites) and a promoter element (SEQ ID NO:4), was amplified by PCR using rat genomic DNA as template and the following oligonucleotides primers:

5'-GATCACCGGTAAGCTTCCACAAGTGCATTTAGCC-3', 42.2.1 (PinAI site underlined) (SEQ ID NO:19) and

5'-GATCACCGGTCTGTAGGTATCTGGACCTCACTG-3', 42.2.2 (SEQ ID NO:20)

or primers:

5'-GATCCGGCCGAAGCTTCCACAAGTGCATTTAGCC-3', 42.2.3 (EagI site underlined) (SEQ ID NO:21) and

5'-GATCCGGCCGCTGTAGGTATCTGGACCTCACTG-3'. 42.2.4 (SEQ ID NO:22)

The oligonucleotides created a unique PinAI (AgiI) site (A/CCGGT) or EagI site (C/GGCCG) at both ends of the PCR fragments. The PCR fragments were ligated into the pGEM-T vector (Promega) to generate plasmids CN249 and CN250. Similarly, CN256 was created using the same strategy but the PB-TRE fragment was ligated into the pCRT vector (Invitrogen); CN271 is identical to CN250 but with a HindIII site at the 5'-end.

These plasmids provide the PB-TRE DNA fragments for the constructs reported below. In some of the adenovirus vectors described below, the endogenous (adenoviral) TREs were not deleted; rather, in each construct, the PB-TRE was inserted between the endogenous TRE (e.g., the E1A TRE) and its respective coding segment (e.g., the E1A coding segment). In other vectors, the endogenous (Ad5) promoter-enhancer has been deleted, and the prostate-specific promoter-enhancer placed immediately upstream of an early gene.

PB-TRE-driven E1A Ad5 plasmid (CN251)

An adenovirus vector in which expression of early gene E1A is under transcriptional control of PB-TRE was constructed as follows.

CN124 was linearized with PinAI and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs). CN249 was digested with PinAI to free the PB-TRE fragment. The PB-TRE fragment was then ligated into the PinAI-linearized CN124, producing CN251. CN253 is similar to CN251 except for the PB-TRE is in the reverse orientation.

Thus, construct CN251 contains the PB-TRE inserted upstream of and operably linked to the E1A coding segment in the Adenovirus 5 genome. The vector CN253 is similar, but the PB-TRE is in the reverse orientation.

PB-TRE-driven E1B Ad5 plasmid (CN254)

An adenovirus derivative in which the expression of early gene E1B is under transcriptional control of the PB-TRE was constructed as follows.

CN124, which carries the left-end of Ad5, as described above, also contains an artificial EagI site at Ad5 nt 1682, or just upstream of the E1B coding segment. The PB-TRE fragment was excised from CN250 with EagI and inserted into CN124 digested with EagI. This produced CN254, which contains the PB-TRE immediately upstream of and operably linked to the E1B coding segment.

CN255 is identical to CN254, but the orientation of the PB-TRE insert is reversed. CN275 is the same as CN254, but with a HindIII site at the 5'-end.

2.2 *Generation of adenovirus constructs in which expression of one adenovirus replication gene is controlled by a PB-TRE and expression of another adenovirus gene is controlled by a PSE-TRE.*

5 *Adenovirus vector comprising a PB-TRE driven E1A and a PSE-TRE driven E1B (CN257)*

An adenovirus vector in which expression of the E1A gene is under control of the PB-TRE and expression of the E1B gene is under control of the prostate specific antigen transcriptional regulatory element (PSE-TRE) was constructed as follows. The PSE-TRE region has been described in detail in, inter alia, U.S. Patent Nos. 5,648,478 and 5,698,443; 10 Lundwall (1989) *Biochim. Biophys. Res. Commun.* 161:1151-1159; and Zhang et al. (1997) *Nucleic Acids Res.* 25:3143-50.

The PinAI PB-TRE fragment was inserted into CN125 digested with PinAI, which cleaves just upstream of E1A, to create construct CN257, which is a plasmid containing a PB-TRE operably linked to the E1A gene and a PSE-TRE operably linked to the E1B 15 gene. CN258 is similar to CN257, but with the opposite orientation of the PB-TRE fragment.

Ad5 plasmid comprising PSE-TRE driven E1A and PB-TRE driven E1B (CN273)

20 An adenovirus vector was constructed in which expression of E1A is mediated by a PSE-TRE and expression of E1B is mediated by a PB-TRE.

CN143 is a pBluescript (Stratagene, La Jolla, California) derivative containing the PSE-TRE fragment. This fragment was excised with PinAI and ligated into PinAI-digested CN254. The final construct is a plasmid containing a PSE-TRE operably linked to the E1A gene and a PB-TRE operably linked to the E1B gene. CN274 is similar to 25 CN273 except for the opposite orientation of PB-TRE.

CN306 was derived from CN124 by removing E1A endogenous promoter of 64 nts. CN321 was created from CN306 with a inserting of large PSE fragment amplified from CN96.

30 CN326 was derived from CN321 by inserting PB-TRE into EagI site. CN321 is a plasmid containing PSE (from CN96) at the PinAI site of CN306.

2.3 Construction of adenovirus constructs in which expression of one adenovirus replication gene is controlled by hKLK2-TRE and expression of another adenovirus replication gene is controlled by PB-TRE

CN463 was generated from CN251 by inserting an *hKLK2*-TRE into the *EagI* site. The *hKLK2*-TRE was excised from plasmid CN446 with *EagI*, ligated into a similarly cut CN251, to produce CN463. The CN463 construct is a plasmid containing a *PB*-TRE immediately upstream and operably linked to the *E1A* gene and an *hKLK2*-TRE (comprising an *hKLK2* enhancer from nucleotides -4814 to -3643 relative to the *hKLK2* transcription start site and minimal *hKLK2* promoter) immediately upstream and operably linked to the *E1B* gene.

2.4 Generation of adenoviruses that contain two heterologous TREs

Adenoviruses that contain two heterologous TREs were generated by homologous recombination in 293 cells. Briefly, 5 µg of CN257 and 5 µg of BHG10, which contains the right hand end of Ad5, was co-transfected into 293 cells. The cells were overlaid with medium, and infectious virus, generated by in vivo recombination, was detected by cytopathic effect and isolated. Plaque-purified stocks of an adenovirus vector, designated CN739, were established. The structure of the recombinant virus was characterized by PCR, restriction endonuclease digestion and Southern blot. The viral genome of CN739 has the *E1A* transcription unit of Ad5 under the control of *PB*-TRE while *E1B* is under the control of *PSE*-TRE.

As shown in Figure 2, adenoviruses CN750, CN753, CN764, CN767, CN770, CN772, CN774, were generated with the same approach except that CN257 was replaced with CN273, CN326, CN416, CN436, CN446, CN459, CN463, respectively.

EXAMPLE 3

In vitro characterization of adenoviral constructs comprising an adenoviral gene under transcriptional control of prostate cell specific heterologous TREs

Plaque assays

To determine whether the adenoviral constructs described in Examples 1 and 2 replicate preferentially in prostate cells, plaque assays were performed. A plaque assay is an infectious quantitative assay that quantifies how efficiently a particular virus produces

an infection in a cell. Plaquing efficiency was evaluated in the following cell types: prostate tumor cell lines (LNCaP, PC-3), breast normal cell line (HBL-100), breast carcinoma cell line (MCF-7), ovarian tumor cell lines (OVCAR-3, SK-OV-3), hepatocarcinoma cell lines (HepG2, SK-Hep-1), and human embryonic kidney cells (293). LNCaP cells express both androgen receptor and PSA, while the other cell lines tested do not. 293 cells serve as a positive control for plaquing efficiency, since this cell line expresses Ad5 E1A and E1B proteins. The plaque assay was performed as follows. Confluent cell monolayers were seeded in 6-well dishes eighteen hours before infection. The monolayers were infected with 10-fold serial dilutions of each virus. After infecting monolayers for four hours in serum-free media (MEM), the media was removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques were scored two weeks after infection. CN702 has no modifications in its E1 region and is used as a wild type control.

Adenovirus with heterologous TREs plaque assay data
(Percent of wild-type adenovirus (PFU/ml))

Table 1:

	293	LNCaP	HBL-100	OVCAR-3	SK-OV-3
Viruses					
CN702	100	100	100	100	100
CN706	100	23	4.2	5.5	8.9
CN764	100	31	0.25	0.032	0.003
CN769	100	11	0.14	0.015	0.0008
CN770	100	24	0.27	0.036	0.084
CN772	100	29	0.27	0.096	0.21

Adenovirus with heterologous TREs plaque assay data
(Percent of wild-type adenovirus (PFU/ml))

Table 2:

	293	LNCaP	HBL-100	OVCAR-3
Viruses				
CN702	100	100	100	100
CN706	100	25	2.7	7.7
CN753	100	33	0.067	0.52
CN755	100	29	0.52	0.6

5

Adenovirus with heterologous TREs plaque assay data
(Percent of wild-type adenovirus (PFU/ml))

Table 3:

	293	LNCaP	HBL-100	OVCAR-3
Viruses				
CN702	100	100	100	100
CN706	100	33	2.5	3.4
CN739	100	35	0.12	0.0023
CN753	100	41	0.23	0.11

10

Adenovirus with heterologous TREs plaque assay data
(Percent of wild-type adenovirus (PFU/ml))

Table 4:

	293	LNCaP	HBL-100	OVCAR-3
Viruses				
CN702	100	100	100	100
CN753	100	37	0.15	0.086

15

CN765	100	20	0.22	0.012
CN772	100	29	0.27	0.096

Adenovirus with heterologous TREs plaque assay data
(Percent of wild-type adenovirus (PFU/ml))

Table 5:

5

	293	LNCaP	HBL-100	OVCAR-3	SK-OV-3
Viruses					
CN702	100	100	100	100	100
CN706	100	23	4.2	6.1	8.9
CN764	100	31	0.25	0.032	0.003
CN769	100	11	0.14	0.015	0.0008
CN770	100	24	0.27	0.036	0.084

Adenovirus with heterologous TREs plaque assay data
(Percent of wild-type adenovirus (PFU/ml))

Table 6:

10

	293	LNCaP	PC-3	HBL-100	OVCAR-3	HepG2	SK-Hep 1
Viruses							
CN702	100	100	100	100	100	100	100
CN706	100	33	74	2.4	1.8	12	1.8
CN739	100	35	46	0.058	0.0057	0.068	0.00

Adenovirus with heterologous TREs plaque assay data
(Percent of wild-type adenovirus (PFU/ml))

Table 7:

15

	293	LNCaP	MCF-7
Viruses			
CN702	100	100	100

CN706	100	25	1.5
CN737	100	26	0.11

Tables 1-7 show the results of plaque assays performed with the adenoviral vectors described in Examples 1 and 2. The results are expressed as percent of wild-type adenovirus plaque-forming units (PFU) per ml. The tables show the average titer of duplicate samples for the viruses tested. The titer for a particular virus in all cell lines was normalized to its titer on 293 cells. Once the titers on a cell type were normalized to 293 cells, the normalized numbers of the recombinant viruses were compared to CN702. A ratio of less than 100 suggests that the virus tested plaques less efficiently than CN702. Conversely, a ratio greater than 100 suggests that the virus plaques more efficiently than CN702.

The following observations were made. First, *hKLK2*-TRE, *PSE*-TRE and *PB*-TRE engineered adenoviruses demonstrate preferential replication in prostate tumor cells. Since this carcinoma expresses androgen receptors, the *PSE*, *hKLK2* and *PB* TREs contained in the adenoviral vectors should be active in promoting the transcription of the adenovirus early genes. The data presented in Tables 1-7 suggest that the heterologous TRE containing adenoviral vectors induce cytopathic effects with a slightly lower efficiency than wild type adenovirus in prostate tumor cells. Second, *hKLK2*-TRE, *PSE*-TRE and *PB*-TRE containing adenoviruses show a dramatically lower plaquing efficiency in non-prostate tumor cells when compared to wild type. For example, in the ovarian carcinoma cell line OVCAR-3, CN764 and CN739 produced 3,000- and 10,000-fold less plaques than wild type Ad5, respectively. The results are similar for these two viruses in HBL-100 cells, where virus replication is also severely compromised. Third, adenoviral vectors containing two prostate cell specific heterologous TREs give 10 to 100-fold (or more) less plaques in non-prostate cells than an adenoviral vector containing a single heterologous prostate cell specific TRE despite the titers of the two types of adenovirus vectors being similar in LNCaP cells. For example, *PSE*-TRE and *hKLK2*-TRE (CN764) or *PSE*-TRE and *PB*-TRE (CN739) adenovirus vectors give 10- to 100-fold less plaques in HBL-100 and OVCAR-3 cells than CN706, although their titers were similar to CN706 in LNCaP cells. Thus, adenoviruses engineered with two different prostate cell specific TREs were significantly attenuated relative to wild-type adenovirus and CN706 in non-prostate cells, but they showed similar activity to CN706 in LNCaPs.

As indicated above, adenoviruses containing two heterologous TREs demonstrated an unexpectedly high preferential replication in prostate tumor cells as compared to wild type adenovirus and to CN706, in which a *PSE*-TRE controls E1A. This increase in specificity is more than an additive effect of inserting a second prostate-specific TRE.

5 Since CN739 and CN764 appeared to be the most specific viruses, they were further characterized in the following experiments.

Cytopathic effects

To characterize the differential viral replication and cytopathic effects (CPE), CPE
10 assays were performed as follows. Cells were infected with virus at increasing multiplicities of infection (MOI) and monitored for cytopathic effect. Assays were terminated when complete cytolysis of the monolayers was observed at an MOI of 0.01 with wild-type adenovirus. One primary, non-immortalized human microvascular endothelial cell line (hMVEC) was chosen to test its sensitivity to CN764 and wild-type
15 adenovirus (CN702) infection, *in vitro*. As shown in Figure 3, CN702 caused complete monolayer cytolysis of hMVECs at MOIs as low as 0.01 within 10 days. In contrast, CN764 infected hMVEC monolayers did not show significant cytopathic effects at the same time points with MOIs of 10, 1.0, 0.1 and 0.01. Cytolysis of hMVECs equivalent to that seen with wild-type adenovirus was only evident at MOIs between 100 and 1000
20 times as high (MOI>10).

When the CPE assay was performed with CN739, the results were similar to that of CN764 as CN739-infected hMVEC monolayers did not show significant cytopathic effects at the same time points with MOIs of 10, 1.0, 0.1 and 0.01.

25 Thus, CN764 and CN739-mediated cytolysis is significantly attenuated relative to wild-type adenovirus in primary normal human cells.

Differential viral replication

To determine if levels of virus replication correlate with the cytopathic effects of CN739 in prostate tumor cells or human normal cells, virus replication titration was
30 carried out on PSA producing prostate tumor cells (LNCaP) and primary human microvascular endothelial cells (hMVECs). Cells were grown to 70-90% confluence and infected with either wild-type adenovirus (CN702) or CN739, CN764, CN765, CN770 for 90 min at a MOI of 10. Fifty-five hours after infection, the virus was released from the

cells by three freeze/thaw cycles, and the resulting supernatant was titered on 293 cells. The amount of CN739 produced 56 hours after infection was normalized against the amount of wild-type virus produced in the same cell line during the same time period. The data, shown in Figure 4, indicate that the titers of adenovirus vectors containing two different prostate cell specific TREs were 30% of CN702 titers in LNCaPs, but were reduced to less than 1/100 those of the wild-type viruses in normal cells. These data suggest that CN764-like viruses replicate poorly in primary normal human cells, and are somewhat attenuated in prostate cancer cells.

One-step growth curve

The efficiency and kinetics of replication of CN739, CN706 and CN702 were measured using a one-step growth curve assay in LNCaPs and hMVECs. Replicate monolayers of LNCaPs and hMVECs were infected at a MOI of 10 to obtain a synchronous infection of all the cells. Duplicate cultures were harvested at various times post-infection. The number of infectious virus was determined by plaque assay on 293 cells (Figure 5). CN739 and CN706 grew at a similar efficiency in LNCaPs. However, under identical conditions, CN739 grew poorly in the hMVECs, producing about 10,000-fold and 80,000-fold less infectious virus than CN706 and wild-type adenovirus, respectively.

Thus, the one-step growth curve demonstrated that an adenovirus containing two different prostate cell specific TREs, each controlling a different adenoviral gene, grew well in prostate tumor cells but grew poorly in non-prostate endothelial cells, indicating significantly enhanced specificity for target cells.

Stability of the adenoviruses containing two different heterologous TREs

The use of two different heterologous TREs in the adenovirus vectors appears to provide stability to the genome during adenoviral replication. CN739 and CN764 were plaque purified for three times and their DNA were examined by PCR, as well as, Southern blot analysis. By this analysis, the viral genomes do not appear to have undergone sequence rearrangement or loss since the fragments were of the expected sizes. The results indicate that the adenoviral genome of the adenovirus vectors containing two different heterologous TREs are stable.

EXAMPLE 4*Testing cytotoxic ability of adenovirus vectors on tumor xenografts*

An especially useful objective in the development of target cell specific adenoviral vectors is to treat patients with neoplasia comprising the target cells. For example, the prostate cell specific adenoviral vectors described above may be useful to treat patients with prostate carcinoma. An initial indicator of the feasibility is to test the vectors using a technique known in the art, such as testing the vectors for cytotoxicity against neoplastic cell, such as prostate carcinoma, xenografts grown subcutaneously in Balb/c nu/nu mice. To examine the therapeutic efficacy of CN739 *in vivo*, LNCaP tumor xenografts were grown in athymic mice. The tumor cells were injected subcutaneously into each flank of each mouse, and after establishment of palpable tumors (mean tumor volume 300 mm³), the tumors were directly injected with CsCl purified CN739 at 2.5×10^8 particles per mm³, or PBS containing 10% glycerol (vehicle) as a control. Tumor growth was then followed for 6 weeks, at which time the mean tumor volume in each group was determined and serum samples were collected for PSA analysis on day 0 and weekly thereafter.

The data depicted in Figure 6 show that treatment of LNCaP tumors with CN739 resulted in an 80% reduction in average tumor volume whereas the average tumor volume in vehicle-treated group I, at day 28, had increased to 400% of the initial volume. Four of seven (57%) animals in CN739-treated group II were free of palpable tumors at day 42. This study demonstrates that a fixed, single dose of an adenovirus containing two different prostate cell specific TREs, each operably linked to two different adenoviral genes, (CN739) per tumor is efficacious against LNCaP xenografts *in vivo*.

Effects on serum PSA levels

The serum PSA level is a widely used marker for the diagnosis and management of prostate carcinoma. LNCaP cells express and secrete high levels of PSA into the culture media and into circulation. An experiment was designed to examine the effects of treatment with CN739 on the serum PSA concentration in mice with LNCaP tumor xenografts.

Following treatment, the average serum PSA level in group I (vehicle only) increased to approximately 800% of the initial value by day 28, whereas the average PSA level in group II (CN706 treatment) and group III (CN739 treatment) remained essentially

constant through day 21 and declined to 10% of the initial value by day 35 (Figure 7). There was a statistically significant difference ($p < 0.001$; T-test) between group I and group II on day 14 and thereafter.

These results demonstrate that CN739 treatment is efficacious in the LNCaP xenograft model when the outcome is measured either by reduction in tumor growth or serum PSA concentration. Taken together with the *in vitro* data, it appears that an adenovirus with two different prostate cell specific TREs (CN739) has a therapeutic efficacy *in vivo* similar to that of an adenovirus with a single prostate cell specific TRE (CN706) but in addition has a higher cell-specificity than CN706.

While it is likely that a therapeutic based on the viruses described here would be given intralesionally (i.e., direct injection), it would also be desirable to determine if intravenous (IV) administration of the virus can affect tumor growth. If so, then it is conceivable that the virus could be used to treat metastatic tumor deposits inaccessible to direct injection. For this experiment, groups of five mice bearing prostate cancer tumors are inoculated with 10^8 pfu of an adenoviral vector of the present invention by tail vein injection, or 10^8 pfu of a replication defective adenovirus (CMV-LacZ) to control for non-specific toxic effects of the virus, or with buffer used to carry the virus. The effect of IV injection of the adenoviral vector on tumor size is compared to the sham treatment.

EXAMPLE 5

Construction of other adenovirus constructs comprising a first adenoviral gene under control of a cell specific heterologous TRE and at least a second gene under control of a second heterologous TRE, where both heterologous TREs are functional in the same cell.

Adenovirus vectors containing heterologous TREs may be generated so as to target the adenoviral replication to a variety of neoplasia. The following describes examples of cell specific (in some cases, tumor cell specific) TREs that may be used to control the expression of adenovirus genes so as to lead to replication of the adenovirus vectors preferentially in the target cells as compared to non-target cells. Cell specific adenovirus vectors are listed under a type of neoplasia for which the vectors may provide a treatment. Several of the adenovirus vectors may be useful in the treatment of more than one type of neoplasia because the TREs are functional in more than one type of tumor cell, as

described herein. Schematic diagrams of exemplary adenovirus vectors are depicted in Fig. 8 (A)-(B).

Generation of liver neoplasia specific adenovirus vectors.

As described above, TREs from the *AFP* gene have been shown to be specifically active in liver neoplasia. Human *AFP* enhancer domains A and B (included in the region -3954 bp to -3335 bp relative to the AFP cap site) were PCR amplified from human genomic DNA using the following primers:

5' GTGACCGGTGCATTGCTGTGAACTCTGTA 3' (39.055B) (SEQ ID NO:23) and
5' ATAAGTGGCCTGGATAAAGCTGAGTGG 3' (39.044D) (SEQ ID NO:24)

The AFP promoter was amplified from -163 to +34 using the following primers:

5' GTCACCGGTCTTTGTTATTGGCAGTGGT 3' (39.055J) (SEQ ID NO:25)

5' ATCCAGGCCACTTATGAGCTCTGTGTCCTT 3' (29.055M) (SEQ ID NO:26)

The enhancer and promoter segments were annealed, and a fusion construct was generated using overlap PCR with primers 39.055B and 39.055J. This minimal enhancer/promoter fragment was digested with *PinA*I and ligated with CN124 using the engineered *Age*I site 5' of the E1A cap site to produce CN219. In CN219, the AFP-TRE is immediately upstream of and operably linked to the E1A coding sequence.

The *AFP*-TRE described above was amplified with the following primers (*Eag*I sites under lined):

5' TATCGGCCGGCGCATTGCTGTGAACTCT 3' (39.077A) (SEQ ID NO:27) and
5' TTACGGCCGCTTTGTTATTGGCAGTG 3' (39.077C) (SEQ ID NO:28)

The PCR product was digested with *Eag*I and ligated into the *Eag*I site immediately upstream of the E1B gene in CN124 to make CN234. In CN234, the AFP-TRE is immediately upstream of and operably linked to the E1B coding sequence.

uPA promoter and the other transcription response element (Riccio et al. (1985) *Nucleic Acids Res.* 13:2759-2771; Cannio et al. (1991) *Nucleic Acids Res.* 19:2303-2308) are amplified by PCR with *Eag*I sites at the ends and ligated into *Eag*I digested CN219, to generate CN479. CN479 is a construct with the AFP-TRE operably linked to E1A and

uPA-TRE operably linked to E1B. Similarly, *uPA*-TRE is engineered into CN234 at the PinAI site, to produce CN480. CN480 is a construct in which E1A is under transcriptional control of *uPA*-TRE and E1B is under transcriptional control of *AFP*-TRE.

5 *Generation of breast cancer cell specific adenovirus vectors.*

As described above, TREs from the *MUC1* gene have been identified that are specifically active in breast cancer cells. An adenovirus vector in which expression of the E1A gene is under control of *MUC1*-TRE was constructed as follows.

10 The *MUC1*-TRE region of SEQ ID NO:29 was amplified from human genomic DNA by PCR with the following primer pairs:

5' TAA TCC GGA CGG TGA CCA CTA GAG GG 3' (39.088A, SEQ ID NO:30) and
5' TAT TCC GGA TCA CTT AGG CAG CGC TG 3' (39.088B, SEQ ID NO:31).

15 The primers were constructed with BspEI ends, which are compatible with the AgeI site in CN124. As described herein, CN124 has an AgeI site at Ad5 nt 547 (just upstream of the E1A transcriptional start at nt 498 and the E1A coding segment beginning with ATG at 610) and an EagI site at Ad5 nt 1682, or just upstream of the E1B coding segment. The *MUC1*-TRE PCR product was digested with BspI and ligated into the AgeI site of CN124 to make CN226. In CN226, the *MUC1*-TRE is immediately upstream of and operably linked to the E1A coding sequence.

20 The *MUC1*-TRE was amplified from CN226 to include EagI ends with the following primer pairs:

5' TAA CGG CCG CGG TGA CCA CTA GAG 3' (39.120A, SEQ ID NO:32) and
5' TAT CGG CCG GCA GAA CAG ATT CAG 3' (39.120B, SEQ ID NO:33).

25 The *MUC1*-TRE PCR product was digested with EagI and ligated in the EagI site CN124 to make CN292. In CN292, the *MUC1*-TRE is immediately upstream of and operably linked to the E1B gene.

30 Human *HER-2/neu (HER)* -TRE (Tal et al. (1987) *Mol. Cell. Biol.* 7:2597-2601; Hudson et al. (1990) *J. Biol. Chem.* 265:4389-4393; Grooteclaes et al. (1994) *Cancer Res.* 54:4193-4199; Ishii et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:4374-4378; Scott et al. (1994) *J. Biol. Chem.* 269:19848-19858) is amplified from human genomic DNA with an EagI site at end, ligated into a EagI cut CN226, to produce CN481. CN481 is a construct in which the *MUC1*-TRE is operably linked to the E1A gene and *HER*-TRE is operably

linked to the E1B gene. Similarly, *HER*-TRE is amplified from CN481 by PCR with PinAI site at the end, and ligated into a similarly cut CN292; to produce CN482. CN482 is a construct in which the *HER*-TRE is operably linked to the E1A gene and the *MUC1*-TRE is operably linked to the E1B gene.

5 Another set of breast cancer cell specific adenoviral vectors is generated when the *uPA*-TRE is released from CN485 with EagI and ligated into a similarly cut CN481, to produce CN487. CN487 is a construct in which the *MUC1*-TRE is operably linked to the E1A gene and the *uPA*-TRE is operably linked to the E1B gene. CN488 is the same as CN487 except the positions of two heterologous TREs are exchanged. CN488 is a
10 construct in which the *uPA*-TRE is operably linked to the E1A gene and the *MUC1*-TRE is operably linked to the E1B gene.

 Another set of breast cancer cell specific adenoviral vectors is generated when the *CEA*-TRE is released from CN484 with EagI and ligated into a similarly cut CN487, to produce CN489. CN489 is a construct in which the *MUC1*-TRE is operably linked to the
15 E1A gene and the *CEA*-TRE is operably linked to the E1B gene. CN490 is the same as CN489 except the positions of two heterologous TREs are exchanged. CN490 is a construct in which the *CEA*-TRE is operably linked to the E1A gene and the *MUC1*-TRE is operably linked to the E1B gene.

Generation of colon cancer cell specific adenovirus vectors.

As described above, TREs from the *CEA* gene have been identified that are specifically active in a number of neoplasia including colon cancers. An adenovirus vector in which expression of the E1A gene is under control of *CEA*-TRE was constructed as follows.

A TRE of the carcinoembryonic antigen (*CEA*-TRE), about -402 to about +69 bp relative to the transcriptional start (SEQ ID NO:7), was amplified by PCR from human genomic DNA using primers(which introduced AgeI sites at the ends):

5' ATT ACC GGT AGC CAC CAC CCA GTG AG 3' (39.174B, SEQ ID NO:34) and
5' TAG ACC GGT GCT TGA GTT CCA GGA AC 3' (39.174D, SEQ ID NO:35).

The *CEA*-TRE PCR fragment was ligated into pGEM-T vector which had been linearized with EcoRV and designated CN265. The *CEA*-TRE was excised from CN265 by digestion with PinAI and was ligated into similarly digested CN124 to generate CN266. CN266 is a construct in which the *CEA*-TRE is operably linked to the E1A gene.

The *CEA*-TRE was amplified from CN266 by PCR using primers:

5' TAA CGG CCG AGC CAC CAC CCA 3' (39.180A, SEQ ID NO:36) and
5' TAT CGG CCG GCT TGA GTT CCA GG 3' (39.180B, SEQ ID NO:37)

The unique restriction site EagI was introduced by the primer pair at the ends of the PCR amplified product. The PCR product was ligated into pGEM-T Vector (Promega), and the resultant plasmid designated CN284. The EagI *CEA*-TRE fragment was excised from CN284 and isolated by gel electrophoresis, and ligated into CN124 which had been cut with EagI to make CN290. CN290 is a construct in which the *CEA*-TRE is immediately upstream of and operably linked to the E1B gene.

A *uPA*-TRE is released from CN479 with EagI and ligated into a similarly cut CN266, to produce CN483. CN483 is a construct in which the *CEA*-TRE is operably linked to the E1A gene and the *uPA*-TRE is operably linked to the E1B gene. Similarly, CN484 is a construct in which the *uPA*-TRE is operably linked to the E1A gene and the *CEA*-TRE is operably linked to the E1B gene.

Generation of colon cancer cell specific adenovirus vectors.

CN485 is a construct in which the *HER*-TRE is operably linked to the E1A gene and the *uPA*-TRE is operably linked to the E1B gene. *uPA*-TRE is released from CN479 with EagI and ligated to a similarly cut CN482, to produce CN485.

CN486 is the same as CN485 except the position of two heterologous TREs are exchanged. CN486 is a construct in which the *uPA*-TRE is operably linked to the E1A gene and the *HER*-TRE is operably linked to the E1B gene.

5 *Generation additional adenovirus vectors containing multiple heterologous TREs.*

The invention does not exclude additional genes under transcriptional control of heterologous TREs. Accordingly, adenovirus vectors may be generated in which a third gene is under transcriptional control of a third heterologous TRE, where all the TREs are different from each other and all are functional in the same cell.

10 An adenovirus vector is generated through the insertion of the *PB*-TRE, the *PSE*-TRE, and the *hKLK2*-TRE in operable linkage with three genes, for example, the E1A, E1B, and E4 genes. These prostate cell specific TREs are amplified as described above and inserted into the adenovirus vectors as described herein. Fig. 9 depicts examples of such adenoviral constructs.

15 Also depicted in Fig. 9 is an example of a construct in which three genes are controlled by three other cell specific TREs described above. The adenovirus construct CN781, for example, may be useful to target liver neoplasia in which the *HER*-TRE is functional.

20 Adenoviruses that contain multiple heterologous TREs are generated by homologous recombination in 293 cells as described above.

EXAMPLE 6

25 *Characterization of an E3 deleted adenovirus, CN751, that contains the adenovirus death protein gene*

30 An Adenovirus death protein mutant, CN751, was constructed to test whether such a construct may be more effective for cytotoxicity. The Adenovirus death protein (ADP), an 11.6 kD Asn-glycosylated integral membrane peptide expressed at high levels late in infection, migrates to the nuclear membrane of infected cells and affects efficient lysis of the host. The Adenovirus 5 (Ad5) E3 region expresses the *adp* gene.

Construction of CN751

CN751 was constructed in two parts. First, an E3 deleted platform plasmid that contains Ad5 sequence 3' from the BamHI site at 21562 bp was generated. The Ad2 *adp* was engineered into the remainder of the E3 region of this plasmid to yield CN252. An ADP cassette is constructed using overlap PCR. The Y leader, an important sequence for correct expression of some late genes, is PCR-amplified using primers:

5' GCCTTAATTAAAAGCAAACCTCACCTCCG...Ad2 28287 bp (37.124.1)
(SEQ ID NO:38); and

5' GTGGAACAAAAGGTGATTAAAAAATCCCAG...Ad2 28622 bp (37.146.1)
(SEQ ID NO:39).

The ADP coding region is PCR amplified using primers:

5' CACCTTTTGTTCACCGCTCTGCTTATTAC...Ad2 29195 bp (37.124.3)
(SEQ ID NO:40) and

5' GGCTTAATTAAGTGTGAAAGGTGGGAGC...Ad2 29872 bp (37.124.4)
(SEQ ID NO:41).

The two fragments were annealed and the overlap product was PCR amplified using primers 37.124.1 and 37.124.4. The ends of the product were polished with Klenow fragment and ligated to BamHI cut pGEM-72 (+) (CN241; Promega, Madison, WI). The ADP cassette was excised by digesting CN241 with Pac 1 restriction endonuclease and ligated with two vectors, CN247 and CN248 generating plasmids CN252 and CN270, respectively. CN247 contains a unique PacI site in the E3 region and was constructed as follows. A plasmid containing the full length Ad5 genome, TG3602 (Transgene, France), was digested with BamHI and religated to yield CN221. The backbone of this plasmid (outside of the Ad5 sequence) contained a PacI site that needed to be removed to enable further manipulations. This was effected by digesting CN221 with PacI and polishing the ends with T4 DNA polymerase, resulting in CN246. CN246 was digested with *AscI* and *AvrII* (to remove intact E3 region). This fragment was replaced by a similarly cut fragment derived from BHG11. The resulting plasmid, CN 247, contained a deleted E3 region and a PacI site suitable for insertion of the ADP cassette fragment (described above). Ligation of CN247 with the ADP cassette generated CN252.

To construct the second part, the 5' Ad5 sequence necessary for CN751 was obtained by digesting purified CN702 DNA with *EcoRI* and isolating the left hand fragment by gel extraction. After digesting CN252 with *EcoRI*, the left hand fragment of

CN702 and CN252 were ligated. 293 cells were transfected with this ligation mixture by using the standard lipofection transfection protocol developed at Calydon, Inc. and incubated at 37°C. Ten days later, the cells were harvested, freeze-thawed three times, and the supernatant was plaqued on 293 monolayers. Individual plaques were picked and used to infect monolayers of 293 cells to grow enough virus to test. After several days, plate lysates were screened using a polymerase chain reaction (PCR) based assay to detect candidate viruses. One of the plaques that scored positive was designated CN751.

Structural Characterization of CN751

The structure of CN751 was confirmed by two methods. First, primers 37.124.1 and 37.124.4 were used to screen candidate viruses by PCR to detect the presence of the *adp* cassette. CN751 produced an extension fragment consistent with the expected product (1065bp). Second, CN751 was analyzed by Southern blot. Viral DNA was purified, digested with *PacI*, *SacI*, and *AccI/XhoI*, and probed with a sequence homologous to the ADP coding region. The structure of CN751 matched the expected pattern.

In Vitro Characterization of CN751

Two experiments were conducted to examine the cytotoxicity and virus yield of CN751. In the first study, CN751's cytotoxicity was evaluated in LNCaP cells by measuring the accumulation of a cytosolic enzyme, lactate dehydrogenase (LDH), in the supernatant over several days. The level of extracellular LDH correlates with the extent of cell lysis. Healthy cells release very little, if any, enzyme, whereas dead cells release large quantities. LDH was chosen as a marker because it is a stable protein that can be readily detected by a simple protocol. CN751's ability to cause cell death was compared to that of CN702, a vector lacking the ADP gene, and Rec700, a vector containing the ADP gene.

Monolayers of LNCaP cells were infected at a multiplicity of infection of one with either CN702, Rec700, or CN751 and then seeded in 96 well dishes. Samples were harvested once a day from one day after infection to five days after infection and scored using Promega's Cytotox 96 kit. This assay uses a coupled enzymatic reaction which converts a tetrazolium salt to a red formazan product that can be determined in a plate reader at 490nm.

Since the absorbance of a sample corresponds to the level of LDH released from infected cells, a plot of how a sample's absorbance changes with time describes how

efficiently the viruses studied induce cell lysis (Figure 10). Each data point represents the average of sixteen separate samples. The results suggest that CN751 kills cells more efficiently than the *adp*- control, CN702, and similarly to the *adp*+ control, Rec700. The concentration of LDH in the supernatant increases rapidly from two days and reaches a maximum at four days in wells infected with CN751. In contrast, LDH concentration in the supernatant of CN702 infected cells begins to rise slowly at two days and continues until the conclusion of the experiment. Significantly, the amount of LDH released from CN751 infected cells at three days is two times that released from CN702 infected cells. The data demonstrate that adenovirus vectors with *adp* gene kill cells more efficiently than adenovirus vectors that lack the *adp* gene.

Not only is it important for adenovirus vectors to kill cells efficiently, they must also be able to shed progeny that can infect other target cells. Viral vectors that can shed large amounts of virus might be better therapeutics than those that shed only small amounts. A virus yield assay was undertaken to evaluate whether CN751 can induce the efficient release of its progeny from the infected cell. A549 cells were infected at an MOI of five. Supernatant was harvested at various times after infection and titered on 293 cells to determine the virus yield (Figure 11). The data suggests that cells infected with CN751 shed virus more efficiently than those infected with CN702. At forty-eight hours post infection, CN751 infected cells have released ten times more virus than CN702 infected. At seventy-two hours post infection, CN751 infected cells have released forty times more virus. In sum, the virus yield data demonstrate that adenovirus vectors with the *adp* gene release more virus.

In Vivo Characterization of CN751

LNCaP nude mouse xenografts were challenged with a single intratumoral dose (1×10^4 particles/mm³ tumor) of either CN751, a vector containing the ADP gene, or CN702, a vector lacking the gene. A third group of tumors was treated with buffer alone. The tumors were monitored weekly for six weeks and their relative volume was graphed against time. The results are shown in Figure 12. Error bars represent the standard error for each sample group. The initial average tumor volume for CN751 treated animals (n = 14) was 320 mm³ for CN702 treated (n = 14), and 343 mm³ for buffer treated (n = 8). The

data suggest that CN751 kills tumor cells more effectively than CN702. On average, tumors challenged with CN751 remained the same size throughout the course of the experiments while nine out of fourteen tumors (64%) regressed. Those treated with CN702 doubled in size. Buffer treated tumors grew to nearly five times their initial volume. The Students T-test indicates that the difference in tumor size between CN751 and CN702 treated tumors was statistically significant from day 7 ($p = 0.016$) through the end of the experiment ($p = 0.003$).

EXAMPLE 7

Preparation of covalently pegylated adenovirus

A series of experiments were carried out to alter the surface capsids of adenovirus by complexing adenovirus with PEG. The objective of PEG complexation masking of the adenovirus surface is the following: 1) adenovirus neutralizing antibodies or opsinins which are in circulation, and 2) increase systemic circulation time of adenovirus particles by reduction of non-specific clearance mechanism in the body (i.e., macrophages, etc.).

Surface capsids of wild type adenovirus CN706 were modified through covalent attachment of PEG to hexon and fiber proteins using N-hydroxysuccinimidyl succinamide (NHS). The PEGs (Shearwater Polymers, Inc.) had nominal molecular weight of 5000 Da. The activated PEG (approximately 2 mM) was reacted with 5×10^9 particles/ml adenovirus in a tris-HCl buffer (the approximate molar ratio of virus particle to PEG was $1:4 \times 10^6$). Various combinations of pH, temperature, and reaction times were used. After the reaction, unreacted activated PEG, unreacted adenovirus, and pegylated adenovirus were separated by anionic ion exchange chromatography on Q Sepharose XL (Pharmacia), running a 0 to 1.5 M NaCl gradient in 50 mM tris, pH 8.0. The gradient was run over 10 column volumes.

Characterization of PEG-CN706

Pegylation of CN706 was verified by SDS-Page. Figure 14 depicts the pegylation of CN706 and the mobility shift of pegylated proteins. Lanes 1 and 2 are non-pegylated CN706 (control), lanes 3 through 6 are pegylated CN706 under several pH and temperature conditions. Lanes 3 through 6 show the appearance of a second band above the hexon proteins of CN706, most likely pegylated hexon, and the loss of the fiber protein band. Since no additional bands associated with the virus except that corresponding to the

PEG-hexon protein, the pegylated fiber protein is assumed to be under one of the unpegylated proteins on the SDS gel.

Figure 15 is an ion exchange chromatogram showing the change in surface properties of CN706. Pegylation of CN706 results in its earlier elution from the Q Sepharose resin used to capture the virus. This result is most likely due to PEG rendering the virus more charge neutral in appearance and hence decreasing its binding potential to the ion exchange matrix. A broadening of the virus' chromatogram is expected since the pegylation of CN706 occurs to different percentages.

The infectivity of pegylated CN706 was evaluated in an *in vitro* plaque assay on 293 cells. Table 8 depicts a 5 to 10-fold reduction in plaquing efficiency of PEG-CN706 as compared to CN706. This is most likely due to pegylation masking the virus cells, decreasing the recognition and endocytosis of the viral particles.

Table 8: Comparison of Plaquing Efficiency of CN706 and PEG-CN706.

Sample Description	Number of Plaques (Arbitrary Units)
CN706	15 ± 5
PEG-CN706	4 ± 1

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Henderson, Daniel R.
Yu, De Chao
- (ii) TITLE OF INVENTION: ADENOVIRUS VECTORS CONTAINING
HETEROLOGOUS TRANSCRIPTION REGULATORY ELEMENTS AND METHODS
OF USING SAME
- (iii) NUMBER OF SEQUENCES: 41
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: MORRISON & FOERSTER
 - (B) STREET: 755 PAGE MILL ROAD
 - (C) CITY: Palo Alto
 - (D) STATE: CA
 - (E) COUNTRY: USA
 - (F) ZIP: 94304-1018
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: Windows
 - (D) SOFTWARE: FastSEQ for Windows Version 2.0b
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
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- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Catherine, Polizzi M
 - (B) REGISTRATION NUMBER: 40,130
 - (C) REFERENCE/DOCKET NUMBER: 34802-20010.00
- (ix) TELECOMMUNICATION INFORMATION:
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 - (C) TELEX: 706141

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5836 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTCTAG	TTTTCTTTTC	CCGGTGACAT	CGTGGAAGC	ACTAGCATCT	CTAAGCAATG	60
ATCTGTGACA	ATATTCACAG	TGTAATGCCA	TCCAGGGAAC	TCAACTGAGC	CTTGATGTCC	120
AGAGATTTTT	GTGTTTTTTT	CTGAGACTGA	GTCTCGCTCT	GTGCCAGGCT	GGAGTGCAGT	180
GGTGCAACCT	TGGCTCACTG	CAAGCTCCGC	CTCTGGGTT	CACGCCATTC	TCCTGCCTCA	240
GCCCTCTGAG	TAGCTGGGAC	TACAGGCACC	CGCCACCACG	CCTGGCTAAT	TTTTTTGTAT	300
TTTGTAGTAGA	GATGGGGTTT	CACTGTGTTA	GCCAGGATGG	TCTCAGTCTC	CTGACCTCGT	360
GATCTGCCCCA	CCTTGGCCTC	CCAAAGTGCT	GGGATGACAG	GCCTGAGCCA	CCGCGCCTGG	420
CCGATATCCA	GAGATTTTTT	GGGGGGCTCC	ATCACACAGA	CATGTTGACT	GTCTTCATGG	480
TTGACTTTTA	GTATCCAGCC	CCTCTAGAAA	TCTAGCTGAT	ATAGTGTGGC	TCAAAACCTT	540
CAGCACAAAT	CACACCGTTA	GACTATCTGG	TGTGGCCCAA	ACCTTCAGGT	GAACAAAGGG	600
ACTCTAATCT	GGCAGGATAT	TCCAAAGCAT	TAGAGATGAC	CTCTTGCAAA	GAAAAAGAAA	660
TGAAAAAGAA	AAAGAAAGAA	AGGAAAAAAA	AAAAAAAAAA	GAGATGACCT	CTCAGGCTCT	720
GAGGGGAAAC	GCCTGAGGTC	TTTGAGCAAG	GTCAGTCCCTC	TGTTGCACAG	TCTCCCTCAC	780
AGGGTCATTG	TGACGATCAA	ATGTGGTCAC	GTGTATGAGG	CACCAGCACA	TGCTGGGCTC	840
TGGGGAGTGC	CGTGTAAGTG	TATGCTTGCA	CTGCTGAATG	CTTGGGATGT	GTGAGGGATT	900
ATCTTCAGCA	CTTACAGATG	CTCATCTCAT	CCTCACAGCA	TCACTATGGG	ATGGGTATTA	960
CTGGCCTCAT	TTGATGGAGA	AAGTGGCTGT	GGCTCAGAAA	GGGGGGACCA	CTAGACCAGG	1020
GACACTCTGG	ATGCTGGGGA	CTCCAGAGAC	CATGACCACT	CACCAACTGC	AGAGAAATTA	1080
ATTGTGGCCT	GATGTCCCTG	TCCTGGAGAG	GGTGGAGGTG	GACCTTCACT	AACCTCCTAC	1140
CTTGACCCTC	TCCTTTAGGG	CTCTTTCTGA	CCTCCACCAT	GGTACTAGGA	CCCCATTGTA	1200
TTCTGTACCC	TCCTGACTCT	ATGACCCCCA	CTGCCCACTG	CATCCAGCTG	GGTCCCCTCC	1260
TATCTCTATT	CCCAGCTGGC	CAGTGCAGTC	TCAGTGCCCA	CCTGTTTGTC	AGTAACTCTG	1320
AAGGGGCTGA	CTTTTACTG	ACTTGCAAAC	AAATAAGCTA	ACTTTCCAGA	GTTTTGTGAA	1380
TGCTGGCAGA	GTCCATGAGA	CTCCTGAGTC	AGAGGCAAAAG	GCTTTTACTG	CTCACAGCTT	1440
AGCAGACAGC	ATGAGGTTCA	TGTTACATT	AGTACACCTT	GCCCCCCCCA	AATCTTGTAG	1500
GGTGACCAGA	GCAGTCTAGG	TGGATGCTGT	GCAGAAGGGG	TTTGTGCCAC	TGGTGAGAAA	1560
CCTGAGATTA	GGAAATCCTCA	ATCTTATACT	GGGACAACCTT	GCAAACCTGC	TCAGCCTTTG	1620
TCTCTGATGA	AGATATTATC	TTCATGATCT	TGGATTGAAA	ACAGACCTAC	TCTGAGGAA	1680
CATATTGTAT	CGATTGTCCT	TGACAGTAAA	CAATCTGTT	GTAAGAGACA	TTATCTTTAT	1740
TATCTAGGAC	AGTAAGCAAG	CCTGGATCTG	AGAGAGATAT	CATCTTGCAA	GGATGCCTGC	1800
TTTACAAACA	TCCTTGAAAC	AACAATCCAG	AAAAAAAAG	GTGTTGCTGT	CTTTGCTCAG	1860
AAGACACACA	GATACGTGAC	AGAACCATGG	AGAATTGCCT	CCCAACGCTG	TTCAGCCAGA	1920
GCCTTCCACC	CTTGTCTGCA	GGACAGTCTC	AACGTTCCAC	CATTAAATAC	TTCTTCTATC	1980
ACATCCTGCT	TCCTTATGCC	TAACCAAGGT	TCTAGGTCCC	GATCGACTGT	GTCTGGCAGC	2040
ACTCCACTGC	CAAAACCAGA	ATAAGGCAGC	GCTCAGGATC	CCGAAGGGGC	ATGGCTGGGG	2100
ATCAGAACTT	CTGGGTTTGA	GTGAGGAGTG	GGTCCACCCT	CTTGAATTTT	AAAGGAGGAA	2160
GAGGCTGGAT	GTGAAGGTAC	TGGGGGAGGG	AAAGTGTGAG	TTCCGAACTC	TAGGTCAT	2220
GAGGGAGGAG	ACTGGTAAGG	TCCCAGCTCC	CGAGGTACTG	ATGTGGGAAT	GGCCTAAGAA	2280
TCTCATATCC	TCAGGAAGAA	GGTGCTGGAA	TCCTGAGGGG	TAGAGTTCTG	GGTATATTTG	2340
TGGCTTAAGG	CTCTTTGGCC	CCTGAAGGCA	GAGGCTGGAA	CCATTAGGTC	CAGGGTTTGG	2400
GGTGATAGTA	ATGGGATCTC	TTGATTCCTC	AAGAGTCTGA	GGATCGAGGG	TTGCCCATTC	2460
TTCCATCTTG	CCACCTAATC	CTTACTCCAC	TTGAGGGTAT	CACCAGCCCT	TCTAGCTCCA	2520
TGAAGGTCCC	CTGGGCAAGC	ACAATCTGAG	CATGAAAGAT	GCCCCAGAGG	CCTTGGGTGT	2580
CATCCACTCA	TCATCCAGCA	TCACACTCTG	AGGGTGTGGC	CAGCACCATG	ACGTCATGTT	2640

GCTGTGACTA	TCCCTGCAGC	GTGCCTCTCC	AGCCACCTGC	CAACCGTAGA	GCTGCCCCATC	2700
CTCCTCTGGT	GGGAGTGGCC	TGCATGGTGC	CAGGCTGAGG	CCTAGTGTCA	GACAGGGAGC	2760
CTGGAATCAT	AGGGATCCAG	GACTCAAAAG	TGCTAGAGAA	TGGCCATATG	TCACCATCCA	2820
TGAAATCTCA	AGGGCTTCTG	GGTGGAGGGC	ACAGGGACCT	GAACCTATGG	TTTCCCAAGT	2880
CTATTGCTCT	CCCAAGTGAG	TCTCCCAGAT	ACGAGGCACT	GTGCCAGCAT	CAGCCTTATC	2940
TCCACCACAT	CTTGTA AAAAG	GACTACCCAG	GGCCCTGATG	AACACCATGG	TGTGTACAGG	3000
AGTAGGGGGT	GGAGGCACGG	ACTCCTGTGA	GGTCACAGCC	AAGGGAGCAT	CATCATGGGT	3060
GGGGAGGAGG	CAATGGACAG	GCTTGAGAAC	GGGGATGTGG	TTGTATTTGG	TTTTCTTTGG	3120
TTAGATAAAG	TGCTGGGTAT	AGGATTGAGA	GTGGAGTATG	AAGACCAGTT	AGGATGGAGG	3180
ATCAGATTGG	AGTTGGGTTA	GATAAAGTGC	TGGGTATAGG	ATTGAGAGTG	GAGTATGAAG	3240
ACCAGTTAGG	ATGGAGGATC	AGATTGGAGT	TGGGTTAGAG	ATGGGGTAAA	ATTGTGCTCC	3300
GGATGAGTTT	GGGATTGACA	CTGTGGAGGT	GGTTTGGGAT	GGCATGGCTT	TGGGATGGAA	3360
ATAGATTTGT	TTTGATGTTG	GCTCAGACAT	CCTTGGGGAT	TGAACTGGGG	ATGAAGCTGG	3420
GTTTGATTTT	GGAGGTAGAA	GACGTGGAAG	TAGCTGTCAG	ATTTGACAGT	GGCCATGAGT	3480
TTTGTTTGAT	GGGGAATCAA	ACAATGGGGG	AAGACATAAG	GGTTGGCTTG	TTAGGTTAAG	3540
TTGCGTTGGG	TTGATGGGGT	CGGGGCTGTG	TATAATGCAG	TTGGATTGGT	TTGTATTAAA	3600
TTGGGTTGGG	TCAGGTTTTG	GTTGAGGATG	ATGCTTGGGG	ACACCGGATC	3660	
CATGAGGTTT	TCAGTGGAGT	GGAGACAAAC	TTCTTTTCCA	GGATGAATCC	AGGGAAGCCT	3720
TAATTCACGT	GTAGGGGAGG	TCAGGCCACT	GGCTAAGTAT	ATCCTTCCAC	TCCAGCTCTA	3780
AGATGGTCTT	AAATTGTGAT	TATCTATATC	CACCTCTGTC	TCCCTCACTG	TGCTTGGAGT	3840
TTACCTGATC	ACTCAACTAG	AAACAGGGGA	AGATTTTATC	AAATTCTTTT	TTTTTTTTTT	3900
TTTTTTTTGA	GACAGAGTCT	CACTCTGTTG	CCCAGGCTGG	AGTGCAGTGG	CGCAGTCTCG	3960
GCTCACTGCA	ACCTCTGCCT	CCCAGGTTCA	AGTGATTCTC	CTGCCTCAGC	CTCCTGAGTT	4020
GCTGGGATTG	CAGGCATGCA	GCACCATGCC	CAGCTAATTT	TTGTATTTTT	AGTAGAGATG	4080
GGGTTTCACC	AATGTTTGCC	AGGCTGGCCT	CGAACTCCCT	ACCTGGTGAT	CCACCTGCCT	4140
CAGCCTCCCA	AAGTGCTGGG	ATTACAGGCG	TCAGCCACCG	CGCCAGCCA	CTTTTGTCAA	4200
ATTCTTGAGA	CACAGCTCGG	GCTGGATCAA	GTGAGCTACT	CTGGTTTTAT	TGAACAGCTG	4260
AAATAACCAA	CTTTTTTGAA	ATTGATGAAA	TCTTACGGAG	TTAACAGTGG	AGGTACCAGG	4320
GCTCTTAAGA	GTTCCCGATT	CTCTTCTGAG	ACTACAAATT	GTGATTTTGC	ATGCCACCTT	4380
AATCTTTTTT	TTTTTTTTTT	TAAATCGAGG	TTTCAGTCTC	ATTCTATTTT	CCAGGCTGGA	4440
GTCTCAATAG	CTCAGTACAG	CTCACTGTAG	CCTTGAATCT	CTGGCCTTAA	GAGATTCTCC	4500
TGCTTCGGTC	TCCCAATAGC	TAAGACTACA	CGAATCCACC	ACCATATCCA	GATAATTTTT	4560
AAATTTTTTG	GGGGGCCGGG	CACAGTGGCT	CACGCCTGTA	ATCCCAACAC	CATGGGAGGC	4620
TGAGATGGGT	GGATCACGAG	GTCAGGAGTT	TGAGACCAGC	CTGACCAACA	TGGTGAAACT	4680
CTGTCTCTAC	TAAAAA AAAA	AAAAATAGAA	AAATTAGCCG	GGCGTGGTGG	CACACGGCAC	4740
CTGTAATCCC	AGCTACTGAG	GAGGCTGAGG	CAGGAGAATC	ACTTGAACCC	AGAAGGCAGA	4800
GGTTGCAATG	AGCCGAGATT	GCGCCACTGC	ACTCCAGCCT	GGGTGACAGA	GTGAGACTCT	4860
GTCTCAAAAA	AAAAAAATTT	TTTTTTTTTT	TTTGTAGAGA	TGGATCTTGC	TTTGTTTCTC	4920
TGGTTGGCCT	TGAACTCCTG	GCTTCAAGTG	ATCCTCCTAC	CTTGGCCTCG	GAAAGTGTTG	4980
GGATTACAGG	CGTGAGCCAC	CATGACTGAC	CTGTCTGTAA	TCTTGAGGTA	CATAAACCTG	5040
GCTCCTAAAG	GCTAAAGGCT	AAATATTTGT	TGGAGAAGGG	GCATTGGATT	TTGCATGAGG	5100
ATGATTCTGA	CCTGGGAGGG	CAGGTCAGCA	GGCATCTCTG	TTGCACAGAT	AGAGTGATCA	5160
GGTCTGGAGA	ACAAGGAGTG	GGGGGTTATT	GGAATTCCAC	ATTGTTTGCT	GCACGTTGGA	5220
TTTTGAAATG	CTAGGGA ACT	TTGGGAGACT	CATATTTCTG	GGCTAGAGGA	TCTGTGGACC	5280
ACAAGATCTT	TTTATGATGA	CAGTAGCAAT	GTATCTGTGG	AGCTGGATTG	TGGGTGGGGA	5340
GTGCAAGGAA	AAGAATGTAC	TAAATGCCAA	GACATCTATT	TCAGGAGCAT	GAGGAATAAA	5400
AGTTCTAGTT	TCTGGTCTCA	GAGTGGTGCA	GGGATCAGGG	AGTCTCACAA	TCTCCTGAGT	5460
GCTGGTGTCT	TAGGGCACAC	TGGGTCTTGG	AGTGCAAAGG	ATCTAGGCAC	GTGAGGCTTT	5520
GTATGAAGAA	TCGGGGATCG	TACCCACCCC	CTGTTTCTGT	TTCATCCTGG	GCATGTCTCC	5580
TCTGCCTTTG	TCCCCTAGAT	GAAGTCTCCA	TGAGCTACAA	GGGCCTGGTG	CATCCAGGGT	5640
GATCTAGTAA	TTGCAGAACA	GCAAGTGCTA	GCTCTCCCTC	CCCTTCCACA	GCTCTGGGTG	5700
TGGGAGGGGG	TTGTCCAGCC	TCCAGCAGCA	TGGGGAGGGC	CTTGCTCAGC	CTCTGGGTGC	5760

CAGCAGGGCA GGGGCGGAGT CCTGGGGAAT GAAGGTTTTA TAGGGCTCCT GGGGGAGGCT 5820
 CCCCAGCCCC AAGCTT 5836

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5835 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAGCTTCTAG	TTTTCTTTTC	CCGGTGACAT	CGTGGAAAGC	ACTAGCATCT	CTAAGCAATG	60
ATCTGTGACA	ATATTACAG	TGTAATGCCA	TCCAGGGAAC	TCAACTGAGC	CTTGATGTCC	120
AGAGATTTT	GTGTTTTTT	CTGAGACTGA	GTCTCGCTCT	GTGCCAGGCT	GGAGTGCAGT	180
GGTGCAACCT	TGGCTCACTG	CAAGCTCCGC	CTCCTGGGTT	CACGCCATT	TCCTGCCTCA	240
GCCTCCTGAG	TAGCTGGGAC	TACAGGCACC	CGCCACCACG	CCTGGCTAAT	TTTTTTGTAT	300
TTTGTAGTAG	GATGGGGTTT	CACCTGTGTTA	GCCAGGATGG	TCTCAGTCTC	CTGACCTCGT	360
GATCTGCCCC	CCTTGGCCTC	CCAAAGTGCT	GGGATGACAG	GCGTGAGCCA	CCGCGCCTGG	420
CCGATATCCA	GAGATTTTTT	GGGGGGCTCC	ATCACACAGA	CATGTTGACT	GTCTTCATGG	480
TTGACTTTTA	GTATCCAGCC	CCTCTAGAAA	TCTAGCTGAT	ATAGTGTGGC	TCAAACCTT	540
CAGCACAAT	CACACCGTTA	GACTATCTGG	TGTGGCCCAA	ACCTTCAGGT	GAACAAAGGG	600
ACTCTAATCT	GGCAGGATAC	TCCAAGCAT	TAGAGATGAC	CTCTTGCAAA	GAAAAAGAAA	660
TGGAAGAGAA	AAAGAAAGAA	AGGAAAAAAA	AAAAAAGAAA	GAGATGACCT	CTCAGGCTCT	720
GAGGGGAAAC	GCCTGAGGTC	TTTGAGCAAG	GTCAGTCCTC	TGTTGCACAG	TCTCCCTCAC	780
AGGGTCATTG	TGACGATCAA	ATGTGGTCAC	GTGTATGAGG	CACCAGCACA	TGCCTGGCTC	840
TGGGGAGTGC	CGTGTAAGTG	TATGCTTGCA	CTGCTGAATG	GCTGGGATGT	GTCAGGGATT	900
ATCTTCAGCA	CTTACAGATG	CTCATCTCAT	CCTCACAGCA	TCACTATGGG	ATGGGTATTA	960
CTGGCCTCAT	TTGATGGAGA	AAGTGGCTGT	GGCTCAGAAA	GGGGGGACCA	CTAGACCAGG	1020
GACACTCTGG	ATGCTGGGGA	CTCCAGAGAC	TAGACCACT	CACCAACTGC	AGAGAAATTA	1080
ATTGTGGCCT	GATGTCCCTG	TCCTGGAGAG	GGTGAGGTG	GACCTTCACT	AACCTCCTAC	1140
CTTGACCCTC	TCTTTTAGGG	CTCTTTCTGA	CCTCCACCAT	GGTACTAGGA	CCCCATTGTA	1200
TTCTGTACCC	TCTTGACTCT	ATGACCCCCA	CGCCCACTG	CATCCAGCTG	GGTCCCCTCC	1260
TATCTCTATT	CCCAGCTGGC	CAGTGCAGTC	TCAGTGCCCA	CCTGTTTGTC	AGTAACTCTG	1320
AAGGGGCTGA	CATTTTACTG	ACTTGCAAA	AAATAAGCTA	ACTTTCCAGA	GTTTTGTGAA	1380
TGCTGGCAGA	GTCCATGAGA	CTCCTGAGTC	AGAGGCAAG	GCTTTTACTG	CTCACAGCTT	1440
AGCAGACAGC	ATGAGGTTCA	TGTTACATT	AGTACACCTT	GCCCCCCCCA	AATCTTGTAG	1500
GGTGACCAGA	GCAGTCTAGG	TGGATGCTGT	GCAGAAGGGG	TTTGTGCCAC	TGGTGAGAAA	1560
CCTGAGATTA	GGAATCCTCA	ATCTTATACT	GGGACAACTT	GCAAACTGTC	TCAGCCTTTG	1620
TCTCTGATGA	AGATATTATC	TTCATGATCT	TGGATTGAAA	ACAGACCTAC	TCTGGAGGAA	1680
CATATTGTAT	CGATTGTCC	TGACAGTAAA	CAAATCTGTT	GTAAGAGACA	TTATCTTTAT	1740
TATCTAGGAC	AGTAAGCAAG	CCTGGATCTG	AGAGAGATAT	CATCTTGCAA	GGATGCCTGC	1800
TTTACAAACA	TCCTTGAAAC	AACAATCCAG	AAAAAAGAG	GTGTTACTGT	CTTTGCTCAG	1860
AAGACACACA	GATACGTGAC	AGAACCATGG	AGAATTGCCT	CCCAACGCTG	TTCAGCCAGA	1920
GCCTTCCACC	CTTTCTGCAG	GACAGTCTCA	ACGTTCCACC	ATTAAATACT	TCTTCTATCA	1980
CATCCCGCTT	CTTTATGCCT	AACCAAGGTT	CTAGGTCCCG	ATCGACTGTG	TCTGGCAGCA	2040
CTCCACTGCC	AAACCCAGAA	TAAGGCAGCG	CTCAGGATCC	CGAAGGGGCA	TGGCTGGGGA	2100
TCAGAACTTC	TGGGTTTGAG	TGAGGAGTGG	GTCCACCCTC	TTGAATTTCA	AAGGAGGAAG	2160
AGGCTGGATG	TGAAGTACT	GGGGGAGGGA	AAGTGTGAGT	TCCGAACCTC	TAGGTCAATG	2220
AGGGAGGAGA	CTGGTAAGGT	CCCAGCTCCC	GAGGTACTGA	TGTGGGAATG	GCCTAAGAA	2280

CTCATATCCT	CAGGAAGAAG	GTGCTGGAAT	CCTGAGGGGT	AGAGTTCTGG	GTATATTTGT	2340
GGCTTAAGGC	TCTTTGGCCC	CTGAAGGCAG	AGGCTGGAAC	CATTAGGTCC	AGGGTTTGGG	2400
GTGATAGTAA	TGGGATCTCT	TGATTCCTCA	AGAGTCTGAG	GATCGAGGGT	TGCCCATTCT	2460
TCCATCTTGC	CACCTAATCC	TTACTCCACT	TGAGGGTATC	ACCAGCCCTT	CTAGCTCCAT	2520
GAAGGTCCCC	TGGGCAAGCA	CAATCTGAGC	ATGAAAGATG	CCCCAGAGGC	CTTGGGTGTC	2580
ATCCACTCAT	CATCCAGCAT	CACACTCTGA	GGGTGTGGCC	AGCACCATGA	CGTCATGTTG	2640
CTGTGACTAT	CCCTGCAGCG	TGCCTCTCCA	GCCACCTGCC	AACCGTAGAG	CTGCCCATCC	2700
TCCTCTGGTG	GGAGTGCCT	GCATGGTGCC	AGGCTGAGGC	CTAGTGTGAG	ACAGGGAGCC	2760
TGGAATCATA	GGGATCCAGG	ACTCAAAAGT	GCTAGAGAAT	GGCCATATGT	CACCATCCAT	2820
GAAATCTCAA	GGGCTTCTGG	GTGGAGGGCA	CAGGGACCTG	AACTTATGGT	TTCCCAAGTC	2880
TATTGCTCTC	CCAAGTGAGT	CTCCAGATA	CGAGGCACTG	TGCCAGCATC	AGCCTTATCT	2940
CCACCACATC	TTGTAAAAGG	ACTACCCAGG	GCCCTGATGA	ACACCATGGT	GTGTACAGGA	3000
GTAGGGGGTG	GAGGCACGGA	CTCCTGTGAG	GTCACAGCCA	AGGGAGCATC	ATCATGGGTG	3060
GGGAGGAGGC	AATGGACAGG	CTTGAGAACG	GGGATGTGGT	TGTATTGGT	TTTCTTTGGT	3120
TAGATAAAGT	GCTGGGTATA	GGATTGAGAG	TGGAGTATGA	AGACCAGTTA	GGATGGAGGA	3180
TCAGATTGGA	GTTGGGTTAG	ATAAAGTGCT	GGGTATAGGA	TTGAGAGTGG	AGTATGAAGA	3240
CCAGTTAGGA	TGGAGGATCA	GATTGGAGTT	GGGTTAGAGA	TGGGGTAAAA	TTGTGCTCCG	3300
GATGAGTTTG	GGATTGACAC	TGTGGAGGTG	GTTTGGGATG	GCATGGCTTT	GGGATGGAAA	3360
TAGATTTGTT	TTGATGTTGG	CTCAGACATC	CTTGGGGATT	GAAGCTGGGA	TGAAGCTGGG	3420
TTTGATTTTG	GAGGTAGAAG	ACGTGGAAGT	AGCTGTCAGA	TTTGACAGTG	GCCATGAGTT	3480
TTGTTTGATG	GGGAATCAAA	CAATGGGGGA	AGACATAAGG	GTTGGCTTGT	TAGGTTAAGT	3540
TGCGTTGGGT	TGATGGGGTC	GGGGCTGTGT	ATAATGCAGT	TGGATTGGTT	TGTATTAAAT	3600
TGGGTTGGGT	CAGGTTTTGG	TTGAGGATGA	GTTGAGGATA	TGCTTGGGGA	CACCGGATCC	3660
ATGAGGTTCT	CACTGGAGTG	GAGACAAACT	TCCTTTCCAG	GATGAATCCA	GGGAAGCCTT	3720
AATTACAGTG	TAGGGGAGGT	CAGGCCACTG	GCTAAGTATA	TCCTTCCACT	CCAGCTCTAA	3780
GATGTTCTTA	AATTGTGATT	ATCTATATCC	ACTTCTGTCT	CCCTCACTGT	GCTTGAGGTT	3840
TACCTGATCA	CTCAACTAGA	AACAGGGGAA	GATTTTATCA	AATTCTTTTT	TTTTTTTTTT	3900
TTTTTTTGAG	ACAGAGTCTC	ACTCTGTTGC	CCAGGCTGGA	GTGCAGTGGC	GCAGTCTCGG	3960
CTCACTGCAA	CCTCTGCCCTC	CCAGGTTCAA	GTGATTCTCC	TGCCTCAGCC	TCCTGAGTTG	4020
CTGGGATTAC	AGGCATGCAG	CACCATGCCC	AGCTAATTTT	TGTATTTTTA	GTAGAGATGG	4080
GGTTTCACCA	ATGTTTGCCA	GGCTGGCCTC	GAACCTCTGA	CCTGGTGATC	CACCTGCCTC	4140
AGCCTCCCAA	AGTGTGGGA	TTACAGGCGT	CAGCCACCGC	GCCCAGCCAC	TTTTGTCAAA	4200
TTCTTTAGAC	ACAGCTCGGG	CTGGATCAAG	ACTGCTACTC	TGGTTTTATT	GAACAGCTGA	4260
AATAACCAAC	TTTTTTGGAAA	TTGATGAAAT	CTTACGGAGT	TAACAGTGGA	GGTACCAGGG	4320
CTCTTAAGAG	TTCCCGATTC	TCTTCTGAGA	CTACAAATTG	TGATTTTGCA	TGCCACCTTA	4380
ATCTTTTTTT	TTTTTTTTTT	AAATCGAGGT	TTCAGTCTCA	TTCTATTTC	CAGGCTGGAG	4440
TTCAATAGCG	TGATCACAGC	TCACTGTAGC	CTTGAATCC	TGGCCTTAAG	AGATTCTCCT	4500
GCTTCGGTCT	CCCAATAGCT	AAGACTACAG	TAGTCCACCA	CCATATCCAG	ATAATTTTTA	4560
AATTTTTTGG	GGGGCCGGGC	ACAGTGGCTC	ACGCCGTGTA	TCCCAACACC	ATGGGAGGCT	4620
GAGATGGGTG	GATCACGAGG	TCAGGAGTTT	GAGACCAGCC	TGACCAACAT	GGTGAAACTC	4680
TGTCTCTACT	AAAAAAAAAA	AAAATAGAAA	AATTAGCCGG	GCGTGGTGGC	ACACGGCACC	4740
TGTAATCCCA	GCTACTGAGG	AGGCTGAGGC	AGGAGAATCA	CTTGAACCCA	GAAGGCAGAG	4800
GTTGCAATGA	GCCGAGATTG	CGCCACTGCA	CTCCAGCCTG	GGTGACAGAG	TGAGACTCTG	4860
TCTCAAAAAA	AAAAAATTTT	TTTTTTTTTT	TTGTAGAGAT	GGATCTTGCT	TTGTTTCTCT	4920
GGTTGGCCTT	GAACCTCTGG	CTTCAAGTGA	TCCTCCTACC	TTGGCCTCGG	AAAGTGTTGG	4980
GATTACAGGC	GTGAGCCACC	ATGACTGACC	TGTCGTTAAT	CTTGAGGTAC	ATAAACCTGG	5040
CTCCTAAAGG	CTAAAGGCTA	AATATTTGTT	GGAGAAGGGG	CATTGGATT	TGCATGAGGA	5100
TGATTCTGAC	CTGGGAGGGC	AGGTCAGCAG	GCATCTCTGT	TGCACAGATA	GAGTGTACAG	5160
GTCTGGAGAA	CAAGGAGTGG	GGGGTTATTG	GAATTCCACA	TTGTTTGCTG	CACGTTGGAT	5220
TTTGAAATGC	TAGGGAACCT	TGGGAGACTC	ATATTTCTGG	GCTAGAGGAT	CTGTGGACCA	5280
CAAGATCTTT	TTATGATGAC	AGTAGCAATG	TATCTGTGGA	GCTGGATTCT	GGGTTGGGAG	5340
TGCAAGGAAA	AGAATGTACT	AAATGCCAAG	ACATCTATTT	CAGGAGCATG	AGGAATAAAA	5400

GTTCTAGTTT	CTGGTCTCAG	AGTGGTGCAT	GGATCAGGGA	GTCTCACAAT	CTCCTGAGTG	5460
CTGGTGTCTT	AGGGCACACT	GGGTCTTGGA	GTGCAAAGGA	TCTAGGCACG	TGAGGCTTTG	5520
TATGAAGAAT	CGGGGATCGT	ACCCACCCCC	TGTTTCTGTT	TCATCCTGGG	CATGTCTCCT	5580
CTGCCTTTGT	CCCCTAGATG	AAGTCTCCAT	GAGCTACAAG	GGCCTGGTGC	ATCCAGGGTG	5640
ATCTAGTAAT	TGCAGAACAG	CAAGTGCTAG	CTCTCCCTCC	CCTTCCACAG	CTCTGGGTGT	5700
GGGAGGGGGT	TGTCCAGCCT	CCAGCAGCAT	GGGGAGGGCC	TTGGTCAGCC	TCTGGGTGCC	5760
AGCAGGGCAG	GGGCGGAGTC	CTGGGGAATG	AAGGTTTTAT	AGGGCTCCTG	GGGGAGGCTC	5820
CCCAGCCCCA	AGCTT					5835

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12047 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTCAGAA	ATAGGGGAAG	GTTGAGGAAG	GACACTGAAC	TCAAAGGGGA	TACAGTGATT	60
GGTTTATTTG	TCTTCTCTTC	ACAACATTGG	TGCTGGAGGA	ATTCCCACCC	TGAGGTTATG	120
AAGATGTCTG	AACACCCAAC	ACATAGCACT	GGAGATATGA	GCTCGACAAG	AGTTTCTCAG	180
CCACAGAGAT	TCACAGCCTA	GGGCAGGAGG	ACACTGTACG	CCAGGCAGAA	TGACATGGGA	240
ATTGCGCTCA	CGATTGGCTT	GAAGAAGCAA	GGACTGTGGG	AGGTGGGCTT	TGTAGTAACA	300
AGAGGGCAGG	GTGAACTCTG	ATTCCCATGG	GGGAATGTGA	TGGTCCTGTT	ACAAATTTTT	360
CAAGCTGGCA	GGGAATAAAA	CCCATTACGG	TGAGGACCTG	TGGAGGGCGG	CTGCCCCAAC	420
TGATAAAGGA	AATAGCCAGG	TGGGGGCCTT	TCCCATTGTA	GGGGGGACAT	ATCTGGCAAT	480
AGAAGCCTTT	GAGACCCTTT	AGGGTACAAG	TACTGAGGCA	GCAAATAAAA	TGAAATCTTA	540
TTTTTCAACT	TTATACTGCA	TGGGTGTGAA	GATATATTTG	TTTCTGTACA	GGGGGTGAGG	600
GAAAGGAGGG	GCAGAGGAAA	TTTCCTGCAG	GTCTGGTTTG	GTCTTGTGAT	CCAGGGGGTC	660
TTGGAAGTAT	TTAAATTAAA	TTAAATTAAA	ACAAGCGACT	GTTTTAAATT	AAATTAAATT	720
AAATTAAATT	TTACTTTATT	TTATCTTAAG	TTCTGGGCTA	CATGTGCAGG	ACGTGCAGCT	780
TTGTTACATA	GGTAAACGTG	TGCCATGGTG	GTTTGCTGTA	CCTATCAACC	CATCACCTAG	840
GTATTAAGCC	CAGCATGCAT	TAGCTGTTTT	TCCTGACGCT	CTCCCTCTCC	CTGACTCCCA	900
CAACAGGCCC	CAGTGTGTGT	TGTTCCCTC	CCTGTGTCCA	TGTGTCTCTCA	TTGTTTCAGCT	960
CCCCTTATA	AGTGAGAACA	TGTGGTGTTT	GGTTTTCTGT	TTCTGTGTTA	GTTTGCTGAG	1020
GATAATGGCT	TCCACCTCCA	TCCATGTTCC	TGCAAAGGAC	GTGATCTTAT	TCTTTTTTAT	1080
GGTTGCATAG	AAATTGTTTT	TACAAATCCA	ATTGATATTG	TATTTAATTA	CAAGTTAATC	1140
TAATTAGCAT	ACTAGAAGAG	ATTACAGAAG	ATATTAGGTA	CATTGAATGA	GGAAATATAT	1200
AAAATAGGAC	GAAGGTGAAA	TATTAGGTAG	GAAAAGTATA	ATAGTTGAAA	GAAGTAAAAA	1260
AAAATATGCA	TGAGTAGCAG	AATGTAAAAG	AGGTGAAGAA	CGTAATAGTG	ACTTTTTAGA	1320
CCAGATTGAA	GGACAGAGAC	AGAAAAATTT	TAAGGAATTG	CTAAACCATG	TGAGTGTTAG	1380
AAGTACAGTC	AATAACATTA	AAGCCTCAGG	AGGAGAAAAG	AATAGGAAAG	GAGGAAATAT	1440
GTGAATAAAT	AGTAGAGACA	TGTTTGATGG	ATTTTAAAAT	ATTTGAAAGA	CCTCACATCA	1500
AAGGATTCAT	ACCGTGCCAT	TGAAGAGGAA	GATGGAAAAG	CCAAGAAGCC	AGATGAAAGT	1560
TAGAAATATT	ATTGGCAAAG	CTTAAATGTT	AAAAGTCCTA	GAGAGAAAAG	ATGGCAGAAA	1620
TATTGGCGGG	AAAGAATGCA	GAACCTAGAA	TATAAATTCA	TCCCAACAGT	TTGGTAGTGT	1680
GCAGCTGTAG	CCTTTTCTAG	ATAATACACT	ATTGTCATAC	ATCGCTTAAG	CGAGTGTAAG	1740
ATGGTCTCCT	CACTTTATTT	ATTTATATAT	TTATTTAGTT	TTGAGATGGA	GCCTCGCTCT	1800
GTCTCCTAGG	CTGGAGTGCA	ATAGTGCAT	ACCACTCACT	GCAACCTCTG	CCTCCTCTGT	1860
TCAAGTGATT	TTCTTACCTC	AGCCTCCCGA	GTAGCTGGGA	TTACAGGTGC	GTGCCACCAC	1920

ACCCGGCTAA	TTTTGTATT	TTTTGTAGAG	ACGGGGTTTT	GCCATGTTGG	CCAGGCTGGT	1980
CTTGAACCTC	TGACATCAGG	TGATCCACCT	GCCTTGGCCT	CCTAAAGTGC	TGGGATTACA	2040
GGCATGAGCC	ACCGTGCCCA	ACCACTTTAT	TTATTTTTTA	TTTTTATTTT	TAAATTCAG	2100
CTTCTATTTG	AAATACAGGG	GGCACATATA	TAGGATTGTT	ACATGGGTAT	ATTGAACCA	2160
GGTAGTGATC	ATACTACCCA	ACAGGTAGGT	TTTCAACCCA	CTCCCCCTCT	TTTCTCCCC	2220
ATTCTAGTAG	TGTGCAGTGT	CTATTGTTCT	CATGTTTATG	TCTATGTGTG	CTCCAGGTTT	2280
AGCTCCCACC	TGTAAGTGAG	AACGTGTGGT	ATTTGATTTT	CTGTCCCTGT	GTAAATTCAC	2340
TTAGGATTAT	GGCTTCCAGC	TCCATTCTATA	TTGCTGTAAA	GGATATGATT	CATTTTTCAT	2400
GGCCATGCAG	TATTCCATAT	TGCGTATAGA	TCACATTTTC	TTTCTTTTTT	TTTTTTGAGA	2460
CGGAGTCTTG	CTTTGCTGCC	TAGGCTGGAG	TGCAGTAGCA	CGATCTCGGC	TCAGTCAAG	2520
CTTACCTCC	GGGGTTCAAG	TCATTCTTCT	GTCTCAGCTT	CCCAAGTAGC	TGGGACTACA	2580
GGCGCCCGCC	ACCACGTCCG	GCTAATTTTT	TTGTGTGTTT	TTAGTAGAGA	TGGGGGTTTC	2640
ACTGTGTTAG	CCAGGATGGT	CTTGATCTCC	TGACCTTGTG	GTCCACCTGC	CTCGGTCTCC	2700
CAAAGTGCTG	GGATTACAGG	GGTGAGCCAC	TGCGCCCGGC	CCATATATAC	CACATTTTCT	2760
TTAACCAATC	CACCATTGAT	GGGCAACTAG	GTAGATTCCA	TGGATTCCAC	AGTTTGTCTA	2820
TTGTGTGCAG	TGTGGCAGTA	GACATATGAA	TGAATGTGTC	TTTTTGGTAT	AATGATTTGC	2880
ATTCCTTTGG	GTATACAGTC	ATTAATAGGA	GTGCTGGGTT	GAACGGTGGC	TCTGTTTAAA	2940
ATTCTTTGAG	AATTTTCCAA	ACTGTTTGCC	ATAGAGAGCA	AACTAATTTA	CATTTCCACG	3000
AACAGTATAT	AAGCATTCCC	TTTTCTCCAC	AGCTTTGTCA	TCATGGTTTT	TTTTTTCTT	3060
TATTTTAAAA	AAGAATATGT	TGTTGTTTTT	CCAGGGTACA	TGTGCAGGAT	GTGCAGGTTT	3120
GTTACATAGG	TAGTAAACGT	GAGCCATGGT	GGTTTGCTGC	ACCTGTCAAC	CCATTACCTG	3180
GGTATGAAGC	CCTGCCTGCA	TAGCTCTTT	TCCCTAATGC	TCTCACTACT	GCCCCACCCT	3240
CACCCTGACA	GGGCAACAG	ACAACCTACA	GAATGGGAGG	AAATTTTTGC	AATCTATTCA	3300
TCTGACAAAG	GTCAAGAATA	TCCAGAATCT	ACAAGGAAT	TAAGCAAAT	TTTACTTTTT	3360
AATAATAGCC	ACTGTACTG	GCGTGAAATG	GTATCTCATT	GTGGTTTTCA	TTTGAATTTT	3420
TCTGATGATC	AGTGACGATG	AGCATTTTTT	CATATTGTGT	GGCTGCTTGT	ACGCTTTTTG	3480
AGAAGTGTCT	CTTCATGCCT	TTTGGCCACT	TTAATGGGAT	TATTTTTTGC	TTTTTAGTTT	3540
AAGTTCCTTA	TAGATTCTGG	ATATTAGACT	TCTTATTGGA	TGCATAGTTT	GTGAATACTC	3600
TCTTCCATTC	TGTAGGTTGT	CTGTTTACTC	TATTGATGGC	TTCTTTTGCT	GTGCCGAAGC	3660
ATCTTAGTTT	AATTAGAAAC	CACCTGCCAA	TTTTTGTGTT	TGTTGCAATT	GCTTTTGGGG	3720
ACTTAGTCA	AAACTCTTTG	CCAAGGTCTG	GGTCAAGAAG	AGTATTTTCT	AGGTTTTCTT	3780
CTAGAATTTT	GAAAGTCTGA	ATGTAAACAT	TTGCATTTT	AATGCATCTT	GAGTTAGTTT	3840
TTGTATATGT	GAAAGGTCTA	CTCTCATTCT	CTTCCCTCT	TTCTTTCTTT	CTTCTTTTTC	3900
TTTCTTTCTT	TCTTTCTTTC	TTTCTTTCTT	TCTTTCTTTC	TTTCTTTTTC	TCCTTCTTTC	3960
TTTCTTTCTT	TCTCTTTCTT	TCTCTTTTTC	TTTTTTTTTT	TTGATGGAGT	ATTGCTCTGT	4020
TGCCCAGGCT	GCAGTGCAGC	GGCACGATCT	CGGCTCACTG	CAACCTCTGC	CTCCTGGGTT	4080
CAACTGATTC	TCCTGCATCA	GCCTTCCAAG	TAGCTGGGAT	TATAGGCGCC	CGCCACCACG	4140
CCCGACTAAT	TTTTGTATTT	TTAGTAGAGA	CGGGGTTGTG	CCATGTTGGC	CAGGCTGGTT	4200
TGAAACTCCT	GACCTCAAAC	GATCTGCCTG	CCTTGGCCTC	CCAAAGTGCT	GGGATTACAG	4260
GTGTGAGCCA	CTGTGCCAG	CCAAGAATGT	CATTTTCTAA	GAGGTCCAAG	AACCTCAAGA	4320
TATTTTGGGA	CCTTGAGAAG	AGAGGAATTC	ATACAGGTAT	TACAAGCACA	GCCTAATGGC	4380
AAATCTTTGG	CATGGCTTGG	CTTCAAGACT	TTAGGCTCTT	AAAAGTCGAA	TCCAAAAATT	4440
TTTATAAAAG	CTCCAGCTAA	GCTACCTTAA	AAGGGGCCCTG	TATGGCTGAT	CACTCTTCTT	4500
GCTATACTTT	ACACAAATAA	ACAGGCCAAA	TATAATGAGG	CCAAAATTTA	TTTGTCAAAT	4560
AAATTGGTCC	TGCTATGATT	TACTCTTGGT	AAGAACAGGG	AAAATAGAGA	AAAATTTAGA	4620
TTGCATCTGA	CCTTTTTTTC	TGAATTTTTA	TATGTGCCTA	CAATTGAGC	TAAATCCTGA	4680
ATTATTTTCT	GGTTGCAAAA	ACTCTCTAAA	GAAGAAGTTG	GTTTTTATTG	TCTTCGTGAC	4740
ACATTTATCT	GGCTCTTTAC	TAGAACAGCT	TTCTGTTTTT	TGGTGTCTTA	GCTTGTGTGC	4800
CTTACAGTTC	TACTCTTCAA	ATTATTGTTA	TGTGTATCTC	ATAGTTTTCC	TTCTTTTGAG	4860
AAAAGTGAAG	CCATGGTATT	CTGAGGACTA	GAGATGACTC	AACAGAGCTG	GTGAATCTCC	4920
TCATATGCAA	TCCACTGGGC	TCGATCTGCT	TCAAATTGCT	GATGCACTGC	TGCTAAAGCT	4980
ATACATTTAA	AACCCTCACT	AAAGGATCAG	GGACCATCAT	GGAAGAGGAG	GAAACATGAA	5040

ATTGTAAGAG	CCAGATTCCG	GGGGTAGAGT	GTGGAGGTCA	GAGCAACTCC	ACCTTGAATA	5100
AGAAGGTAAA	GCAACCTATC	CTGAAAGCTA	ACCTGCCATG	GTGGCTTCTG	ATTAACCTCT	5160
GTTCTAGGAA	GACTGACAGT	TTGGGTCTGT	GTCATTGCC	AAATCTCATG	TTAAATTGTA	5220
ATCCCCAGTG	TTCGGAGGTG	GGACTTGGTG	GTAGGTGATT	CGGTCATGGG	AGTAGATTTT	5280
CTTCTTTGTG	GTGTTACAGT	GATAGTGAGT	GAGTTCTCGT	GAGATCTGGT	CATTTAAAG	5340
TGTGTGGCCC	CTCCCTCCC	TCTCTGGTC	CTCCTACTGC	CATGTAAGAT	ACCTGCTCCT	5400
GCTTTGCCTT	CTACCATAAG	TAAAAGCCCC	CTGAGGCCTC	CCCAGAAGCA	GATGCCACCA	5460
TGCTTCCTGT	ACAGCCTGCA	GAACCATCAG	CCAATTAAAC	CTCTTTTCTG	TATAAATTAC	5520
CAGTCTTGAG	TATCTCTTTA	CAGCAGTGTG	AGAACGGACT	AATACAAGGG	TCTCCAAAT	5580
TCCAAGTTTA	TGTATTCTTT	CTTGCCAAAT	AGCAGGTATT	TACCATAAAT	CCTGTCTTAA	5640
GGTCAAACAA	CCTTGATGGC	ATCGTACTTC	AATTGTCTTA	CACATTCTCT	CTGAATGACT	5700
CCTCCCTAT	GGCATATAAG	CCCTGGGTCT	TGGGGGATAA	TGGCAGAGGG	GTCCACCATC	5760
TTGTCTGGCT	GCCACCTGAG	ACACGGACAT	GGCTTCTGTT	GGTAAGTCTC	TATTAAATGT	5820
TTCTTTCTAA	GAAACTGGAT	TTGTGAGCTT	GTTTCTTTGG	CCTCTCAGCT	TCCTCAGACT	5880
TTGGGGTAGG	TTGCACAACC	CTGCCACCA	CGAAACAAAT	GTTTAATATG	ATAAATATGG	5940
ATAGATATAA	TCCACATAAA	TAAAAGCTCT	TGGAGGGCCC	TCAATAATTG	TTAAGAGTGT	6000
AAATGTGTCC	AAAGATGGAA	AATGTTTGAG	AACTACTGTC	CCAGAGATTT	TCCTGAGTTC	6060
TAGAGTGTGG	GAATATAGAA	CCTGGAGCTT	GGCTTCTTCA	GCCTAGAATC	AGGAGTATGG	6120
GGCTGAAGTC	TGAAGCTTGG	CTTCAGCAGT	TTGGGGTTGG	CTTCCGGAGC	ACATATTTGA	6180
CATGTTGCGA	CTGTGATTTG	GGGTTTGGA	TTTGCTCTGA	ATCCTAATGT	CTGTCCTTGA	6240
GGCATCTAGA	ATCTGAAATC	TGTGGTCAGA	ATTCTATTAT	CTTGAGTAGG	ACATCTCCAG	6300
TCCTGGTTCT	GCCTTCTAGG	GCTGGAGTCT	GTAGTCAGTG	ACCCGGTCTG	GCATTTCAAC	6360
TTCATATACA	GTGGGCTATC	TTTTGGTCCA	TGTTTCAACC	AAACAACCGA	ATAAACCAT	6420
AGAACCTTTC	CCCCTTCCC	TAGCTGCAAT	GTTAAACCTA	GGATTTCTGT	TTAATAGGTT	6480
CATATGAATA	ATTTCAGCCT	GATCCAACCT	TACATTCTCT	CTACCGTTAT	TCTACACCCA	6540
CCTTAAAAAT	GCATTCCCAA	TATATTCCTT	GGATTCTACC	TATATATGGT	AATCCTGGCT	6600
TTGCCAGTTT	CTAGTGCATT	AACATACCTG	ATTTACATT	TTTTACTTTA	AAGTGGAAT	6660
AAGAGTCCCT	CTGCAGAGTT	CAGGAGTTCT	CAAGATGGCC	CTTACTTCTG	ACATCAATTG	6720
AGATTTCAAG	GGAGTCGCCA	AGATCATCCT	CAGGTTTCACT	GATTGCTGGT	AGCCCTCATA	6780
TAACCAATG	AAAGCTGTGA	TGCTCATGGC	TATGGTTTAT	TACAGCAAAA	GAATAGAGAT	6840
GAAAATCTAG	CAAGGGAAGA	GTTGCATGGG	GCAAAGACAA	GGAGAGCTCC	AAGTGCAGAG	6900
ATTCTGTGTT	TTTTCTCCCA	GTGGTGTCT	GGAAAGCAGT	ATCTTCTCCA	TACAATGATG	6960
TGTGATAATA	TTCAGTGTAT	TGCCAATCAG	GGAACTCAAC	TGAGCCTTGA	TTATATTGGA	7020
GCTTGGTTGC	ACAGACATGT	CGACCACCTT	CATGGCTGAA	CTTTAGTACT	TAGCCCCCTC	7080
AGACGTCTAC	AGCTGATAGG	CTGTAACCCA	ACATTGTCAC	CATAAATCAC	ATTGTTAGAC	7140
TATCCAGTGT	GGCCCAAGCT	CCCGTGTA	CACAGGCACT	CTAAACAGGC	AGGATATTTT	7200
AAAAGCTTAG	AGATGACCTC	CCAGGAGCTG	AATGCAAAGA	CCTGGCCTCT	TTGGGCAAGG	7260
AGAATCCTTT	ACCGCACACT	CTCCTTCACA	GGGTTATTGT	GAGGATCAAA	TGTGGTCATG	7320
TGTGTGAGAC	ACCAGCACAT	GTCTGGCTGT	GGAGAGTGAC	TTCTATGTGT	GCTAACATTT	7380
CTGAGTGCTA	AGAAAGTATT	AGGCATGGCT	TTCAGCACTC	ACAGATGCTC	ATCTAATCCT	7440
CACAACATGG	CTACAGGGTG	GGCACTACTA	GCCTCATTTG	ACAGAGGAAA	GGACTGTGGA	7500
TAAGAAGGGG	GTGACCAATA	GGTCAGAGTC	ATTCTGGATG	CAAGGGGCTC	CAGAGGACCA	7560
TGATTAGACA	TTGTCTGCAG	AGAAATTATG	GCTGGATGTC	TCTGCCCCGG	AAAGGGGGAT	7620
GCACTTTCCT	TGACCCCTTA	TCTCAGATCT	TGACTTTGAG	GTTATCTCAG	ACTTCCTCTA	7680
TGATACCAGG	AGCCCATCAT	AATCTCTCTG	TGTCCTCTCC	CCTTCCTCAG	TCTTACTGCC	7740
CACCTCTCCC	AGTCCATCT	CCAGCTGGCC	AGGTGTAGCC	ACAGTACCTA	ACTCTTTGCA	7800
GAGAACTATA	AATGTGTATC	CTACAGGGGA	GAAAAAATAA	AAGAACTCTG	AAAGAGCTGA	7860
CATTTTACCG	ACTTGCAAA	ACATAAGCTA	ACCTGCCAGT	TTTGTGCTGG	TAGAATCAT	7920
GAGACTCCTG	GGTCAGAGGC	AAAAGATTTT	ATTACCCACA	GCTAAGGAGG	CAGCATGAAC	7980
TTTGTGTTCA	CATTTGTTCA	CTTTGCCCCC	CAATTCATAT	GGGATGATCA	GAGCAGTTCA	8040
GGTGGATGGA	CACAGGGGTT	TGTGGCAAAG	GTGAGCAACC	TAGGCTTAGA	AATCCTCAAT	8100
CTTATAAGAA	GGTACTAGCA	AACTTGTTCCA	GTCTTTGTAT	CTGACGGAGA	TATTATCTTT	8160

ATAATTGGGT	TGAAAGCAGA	CCTACTCTGG	AGGAACATAT	TGTATTTATT	GTCCTGAACA	8220
GTAAACAAAT	CTGCTGTAAA	ATAGACGTTA	ACTTTATTAT	CTAAGGCAGT	AAGCAAACCT	8280
AGATCTGAAG	GCGATACCAT	CTTGCAAGGC	TATCTGCTGT	ACAAATATGC	TTGAAAAGAT	8340
GGTCCAGAAA	AGAAAACGGT	ATTATTGCCT	TTGCTCAGAA	GACACACAGA	AACATAAGAG	8400
AACCATGGAA	AATTGTCTCC	CAACACTGTT	CACCCAGAGC	CTTCCACTCT	TGTCTGCAGG	8460
ACAGTCTTAA	CATCCCATCA	TTAGTGTGTC	TACCACATCT	GGCTTCACCG	TGCCTAACCA	8520
AGATTTCTAG	GTCCAGTTCC	CCACCATGTT	TGGCAGTGCC	CCACTGCCAA	CCCCAGAATA	8580
AGGGAGTGCT	CAGAATTCGG	AGGGGACATG	GGTGGGGATC	AGAACTTCTG	GGCTTGAGTG	8640
CAGAGGGGGC	CCATACTCCT	TGGTTCCGAA	GGAGGAAGAG	GCTGGAGGTG	AATGTCCTTG	8700
GAGGGGAGGA	ATGTGGGTTC	TGAACTCTTA	AATCCCCAAG	GGAGGAGACT	GGTAAGGTC	8760
CAGCTTCCGA	GGTACTGACG	TGGGAATGGC	CTGAGAGGTC	TAAGAATCCC	GTATCCTCGG	8820
GAAGGAGGGG	CTGAAATTGT	GAGGGGTGGA	GTTGCAGGGG	TTTGTTAGCT	TGAGACTCCT	8880
TGGTGGGTCC	CTGGGAAGCA	AGGACTGGAA	CCATTGGCTC	CAGGGTTTGG	TGTGAAGGTA	8940
ATGGGATCTC	CTGATTCTCA	AAGGGTCAGA	GGACTGAGAG	TTGCCCATGC	TTTGATCTTT	9000
CCATCTACTC	CTTACTCCAC	TTGAGGGTAA	TCACCTACTC	TTCTAGTTCC	ACAAGAGTGC	9060
GCCTGCGCGA	GTATAATCTG	CACATGTGCC	ATGTCCCAGG	GCCTGGGGCA	TCATCCACTC	9120
ATCATTACAG	ATCTGCGCTA	TGCGGGCGCA	GCCGGCGCCA	TGACGTCATG	TAGCTGCGAC	9180
TATCCCTGCA	GCGCGCCTCT	CCCCTCACGT	CCCAACCATG	GAGCTGTGGA	CGTGCGTCCC	9240
CTGGTGGATG	TGGCCTGCGT	GGTGCCAGGC	CGGGGCCTGG	TGTCCGATAA	AGATCCTAGA	9300
ACCACAGGAA	ACCAGGACTG	AAAGGTGCTA	GAGAATGGCC	ATATGTCGCT	GTCCATGAAA	9360
TCTCAAGGAC	TTCTGGGTGG	AGGGCACAGG	AGCCTGAACT	TACGGGTTTG	CCCCAGTCCA	9420
CTGTCCCTCC	AAGTGAGTCT	CCCAGATACG	AGGCACTGTG	CCAGCATCAG	CTTCATCTGT	9480
ACCACATCTT	GTAACAGGGA	CTACCCAGGA	CCCTGATGAA	CACCATGGTG	TGTGCAGGAA	9540
GAGGGGGTGA	AGGCATGGAC	TCCTGTGTGG	TCAGAGCCCA	GAGGGGGCCA	TGACGGGTGG	9600
GGAGGAGGCT	GTGGACTGGC	TCGAGAAGTG	GGATGTGGTT	GTGTTTGATT	TCCTTTGGCC	9660
AGATAAAGTG	CTGGATATAG	CATTGAAAAC	GGAGTATGAA	GACCAGTTAG	AATGGAGGGT	9720
CAGGTTGGAG	TTGAGTTACA	GATGGGGTAA	AATTCTGCTT	CGGATGAGTT	TGGGGATTGG	9780
CAATCTAAAG	GTGGTTTGGG	ATGGCATGGC	TTTGGGATGG	AAATAGGTTT	GTTTTTATGT	9840
TGGCTGGGAA	GGGTGTGGGG	ATTGAATTGG	GGATGAAGTA	GGTTTAGTTT	TGAGATAGA	9900
ATACATGGAG	CTGGCTATTG	CATGCGAGGA	TGTGCATTAG	TTTGGTTTGA	TCTTTAAATA	9960
AAGGAGGCTA	TTAGGGTTGT	CTTGAATTAG	ATTAAGTTGT	GTTGGGTTGA	TGGGTTGGGC	10020
TTGTGGGTGA	TGTGGTTGGA	TTGGGCTGTG	TGAAATTGGT	TTGGGTCAGG	TTTGGTTGGA	10080
GGTTATCATG	GGGATGAGGA	TATGCTTGGG	ACATGGATTG	AGGTGGTTCT	CATTCAAGCT	10140
GAGGCAAATT	TCCTTTCAGA	CGGTCATTCC	AGGGAACGAG	TGGTTGTGTG	GGGGAATCA	10200
GGCCACTGGC	TGTGAATATC	CCTCTATCCT	GGTCTTGAAT	TGTGATTATC	TATGTCCATT	10260
CTGTCTCCTT	CACGTGACTT	GGAATTGATC	TGGTCATTCA	GCTGGAAATG	GGGGAAGATT	10320
TTGTCAAATT	CTTGAGACAC	AGCTGGGTCT	GGATCAGCGT	AAGCCTTCCT	TCTGGTTTTA	10380
TTGAACAGAT	GAAATCACAT	TTTTTTTTTC	AAAATCACAG	AAATCTTATA	GAGTTAACAG	10440
TGGACTCTTA	TAATAAGAGT	TAACACCAGG	ACTCTTATTC	TTGATTCTTT	TCTGAGACAC	10500
CAAAATGAGA	TTTCTCAATG	CCACCCTAAT	TCTTTTTTTT	TTTTTTTTTT	TTTTTGAGAC	10560
ACAGTCTGGG	TCTTTTGCTC	TGTCACCTAG	GCTGGAGCGC	AGTGGTGTGA	TCATAGCTCA	10620
CTGAACCCCT	GACCTCCTGG	ACTTAAGGGA	TCCTCCTGCT	TCAGCCTCCT	GAGTAGATGG	10680
GGCTACAGGT	GCTTGCCACC	ACACCTGGCT	AATTAATTTT	TTTTTTTTTT	TTTGTAGAGA	10740
AAGGGTCTCA	CTTTGTTGCC	CTGGCTGATC	TTGAACTTCT	GACTTCAAGT	GATTCTTCAG	10800
CCTTGGACTION	CCAAAGCACT	GGGATTGCTG	GCATGAGCCA	CTCACCCTGC	CTGGCTTGCA	10860
GCTTAATCTT	GGAGTGTATA	AACCTGGCTC	CTGATAGCTA	GACATTTTCA	TGAGAAGGAG	10920
GCATTGGATT	TTGCATGAGG	ACAATTCTGA	CCTAGGAGGG	CAGGTCAACA	GGAATCCCCG	10980
CTGTACCTGT	ACGTTGTACA	GGCATGGAGA	ATGAGGAGTG	AGGAGGCCGT	ACCGGAACCC	11040
CATATTGTTT	AGTGGACATT	GGATTTTGAA	ATAATAGGGA	ACTTGGTCTG	GGAGAGTCAT	11100
ATTTCTGGAT	TGGACAATAT	GTGGTATCAC	AAGGTTTAT	GATGAGGGAG	AAATGTATGT	11160
GGGGAACCAT	TTTCTGAGTG	TGGAAGTGCA	AGAATCAGAG	AGTAGCTGAA	TGCCAACGCT	11220
TCTATTTTCA	GAACATGGTA	AGTTGGAGGT	CCAGCTCTCG	GGCTCAGACG	GGTATAGGGA	11280

CCAGGAAGTC	TCACAATCCG	ATCATTCTGA	TATTTTCAGGG	CATATTAGGT	TTGGGGTGCA	11340
AAGGAAGTAC	TTGGGACTTA	GGCACATGAG	ACTTTGTATT	GAAAATCAAT	GATTGGGGCT	11400
GGCCGTGGTG	CTCAGCCTG	TAATCTCATC	ACTTTGGGAG	ACCGAAGTGG	GAGGATGGCT	11460
TGATCTCAAG	AGTTGGACAC	CAGCCTAGGC	AACATGGCCA	GACCTCTCT	CTACAAAAAA	11520
ATTAAAAATT	AGCTGGATGT	GGTGGTGCAT	GCTTGTGGTC	TCAGCTATCC	TGGAGGCTGA	11580
GACAGGAGAA	TCGGTTGAGT	CTGGGAGTTC	AAGGCTACAG	GGAGCTGCGA	TCACGCCGCT	11640
GCACTCCAGC	CTGGGAAACA	GAGTGAGACT	GTCTCAGAAT	TTTTTTAAAA	AAGAATCAGT	11700
GATCATCCCA	ACCCCTGTTG	CTGTTTCATCC	TGAGCCTGCC	TTCTCTGGCT	TTGTTCCCTA	11760
GATCACATCT	CCATGATCCA	TAGGCCCTGC	CCAATCTGAC	CTCACACCGT	GGGAATGCCT	11820
CCAGACTGAT	CTAGTATGTG	TGGAACAGCA	AGTGCTGGCT	CTCCCTCCCC	TTCCACAGCT	11880
CTGGGTGTGG	GAGGGGGTTG	TCCAGCCTCC	AGCAGCATGG	GGAGGGCCTT	GGTCAGCATC	11940
TAGGTGCCAA	CAGGGCAAGG	GCGGGGTCTT	GGAGAATGAA	GGCTTTATAG	GGCTCCTCAG	12000
GGAGGCCCCC	CAGCCCCAAA	CTGCACCACC	TGGCCGTGGA	CACCGGT		12047

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 454 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAGCTTCCAC	AAGTGCATTT	AGCCTCTCCA	GTATTGCTGA	TGAATCCACA	GTTCAGGTTC	60
AATGGCGTTC	AAAACCTGAT	CAAAAATGAC	CAGACTTTAT	ATTCTTACAC	CAACATCTAT	120
CTGATTGGAG	GAATGGATAA	TAGTCATCAT	GTTTAAACAT	CTACCATTCC	AGTTAAGAAA	180
ATATGATAGC	ATCTTGTTCT	TAGTCTTTTT	CTTAATAGGG	ACATAAAGCC	CACAAATAAA	240
AATATGCCTG	AAGAATGGGA	CAGGCATTGG	GCATTGTCCA	TGCCTAGTAA	AGTACTCCAA	300
GAACCTATTT	GTATACTAGA	TGACACAATG	TCAATGTCTG	TGTACAACCTG	CCAACCTGGGA	360
TGCAAGACAC	TGCCCATGCC	AATCATCCTG	AAAAGCAGCT	ATAAAAAGCA	GGAAGCTACT	420
CTGCACCTTG	TCAGTGAGGT	CCAGATACCT	ACAG			454

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5224 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAATTCTTAG	AAATATGGGG	GTAGGGGTGG	TGGTGGTAAT	TCTGTTTTCA	CCCCATAGGT	60
GAGATAAGCA	TTGGGTAAAA	TGTGCTTTCA	CACACACATC	ACATTTTATA	AGAATTAAGG	120
AACAGACTAT	GGGCTGGAGG	ACTTTGAGGA	TGCTGTCTCT	ATAACACTTG	GGTTGTATCT	180
GTTCTATGGG	GCTTGTTTAA	AGCTTGGCAA	CTTGCAACAG	GGTTCACTGA	CTTTCTCCCC	240
AAGCCCAAGG	TACTGTCCTC	TTTTCATATC	TGTTTGGGGG	CCTCTGGGGC	TTGAATATCT	300
GAGAAAATAT	AAACATTTCA	ATAATGTTCT	GTGGTGAGAT	GAGTATGAGA	GATGTGTCAT	360
TCATTGTGAT	CAATGAATGA	ATGAGGACAA	TTAGTGTATA	AATCCTTAGT	ACAACAATCT	420

GAGGGTAGGG	GTGGTACTAT	TCAATTTCTA	TTTATAAAGA	TACTTATTTT	TATTTATTTA	480
TGCTTGTGAC	AAATGTTTTG	TTCGGGACCA	CAGGAATCAC	AAAGATGAGT	CTTTGAATTT	540
AAGAAGTTAA	TGGTCCAGGA	ATAATTACAT	AGCTTACAAA	TGACTATGAT	ATACCATCAA	600
ACAAGAGGTT	CCATGAGAAA	ATAATCTGAA	AGGTTTAAATA	AGTTGTCAAA	GGTGAGAGGG	660
CTCTTCTCTA	GCTAGAGACT	AATCAGAAAT	ACATTCAGGG	ATAATTATTT	GAATAGACCT	720
TAAGGGTTGG	GTACATTTTG	TTCAAGCATT	GATGGAGAAG	GAGAGTGAAT	ATTTGAAAAC	780
ATTTTCAACT	AACCAACCAC	CCAATCCAAC	AAACAAAAAA	TGAAAAGAAT	CTCAGAAACA	840
GTGAGATAAG	AGAAGGAATT	TTCTCACAA	CCACACGTAT	AGCTCAACTG	CTCTGAAGAA	900
GTATATATCT	AATATTTAAC	ACTAACATCA	TGCTAATAAT	GATAATAATT	ACTGTCATTT	960
TTTAATGTCT	ATAAGTACCA	GGCATTTAGA	AGATATTATT	CCATTTATAT	ATCAAAATAA	1020
ACTTGAGGGG	ATAGATCATT	TTCATGATAT	ATGAGAAAAA	TTAAAAACAG	ATTGAATTAT	1080
TTGCCTGTCA	TACAGCTAAT	AATTGACCAT	AAGACAATTA	GATTTAAATT	AGTTTTGAAT	1140
CTTTCTAATA	CCAAAGTTCA	GTTTACTGTT	CCATGTTGCT	TCTGAGTGGC	TTCACAGACT	1200
TATGAAAAAG	TAAACGGAAT	CAGAATTACA	TCAATGCAAA	AGCATTGCTG	TGAACTCTGT	1260
ACTTAGGACT	AAACTTTGAG	CAATAACACA	CATAGATTGA	GGATTGTTTG	CTGTTAGCAT	1320
ACAAACTCTG	GTTCAAAGCT	CCTCTTTATT	GCTTGTCTTG	GAAAATTTGC	TGTTCTTCAT	1380
GGTTTCTCTT	TTCAGTCTA	TCTATTTTTC	TCAACCACTC	ACATGGCTAC	AATAACTGTC	1440
TGCAAGCTTA	TGATTCCCAA	ATATCTATCT	CTAGCCTCAA	TCTTGTTCCT	GAAGATAAAA	1500
AGTAGTATTC	AAATGCACAT	CAACGTCTCC	ACTTGGAGGG	CTTAAAGACG	TTTCAACATA	1560
CAAACCGGGG	AGTTTTGCCT	GGAATGTTTC	CTAAAATGTG	TCCTGTAGCA	CATAGGGTCC	1620
TCTTGTTCCT	TAAAATCTAA	TTACTTTTAG	CCCAGTGTCT	ATCCCACCTA	TGGGGAGATG	1680
AGAGTGAAAA	GGGAGCCTGA	TTAATAATTA	CACTAAGTCA	ATAGGCATAG	AGCCAGGACT	1740
GTTTGGGTAA	ACTGGTCACT	TTATCTTAAA	CTAAATATAT	CCAAAACCTGA	ACATGTACTT	1800
AGTTACTAAG	TCTTTGACTT	TATCTCATTC	ATACCACTCA	GCTTTATCCA	GGCCACTTAT	1860
TTGACAGTAT	TATTGCGAAA	ACTTCCTAAC	TGGTCTCCTT	ATCATAGTCT	TATCCCCTTT	1920
TGAAACAAAA	GAGACAGTTT	CAAAATACAA	ATATGATTTT	TATTAGCTCC	CTTTTGTGTT	1980
CTATAATAGT	CCCAGAAGGA	GTTATAAACT	CCATTTAAAA	AGTCTTTGAG	ATGTGGCCCT	2040
TGCCAACTTT	GCCAGGAATT	CCCAATATCT	AGTATTTTCT	ACTATTAAAC	TTTGTGCCTC	2100
TTCAAACCTG	CATTTTCTCT	CATTCCCTAA	GTGTGCATTG	TTTTCCCTTA	CCGGTTGGTT	2160
TTTCCACCAC	CTTTTACATT	TTCTTGGAAC	ACTATACCCT	CCCTCTTCAT	TTGGCCCACC	2220
TCTAATTTTC	TTTCAGATCT	CCATGAAGAT	GTTACTTCCT	CCAGGAAGCC	TTATCTGACC	2280
CCTCCAAAGA	TTTCATGAGT	TCCTCTTTTC	ATTCTACTAA	TCACAGCATC	CATCACACCA	2340
TGTTGTGATT	ACTGATACTA	TTGTCTGTTT	CTCTGATTAG	GCAGTAAGCT	CAACAAGAGC	2400
TACATGGTGC	CTGTCTCTTG	TTGCTGATTA	TTCCCATCCA	AAAACAGTGC	CTGGAATGCA	2460
GACTTAACAT	TTTATTGAAT	GAATAAATAA	AACCCCATCT	ATCGAGTGCT	ACTTTGTGCA	2520
AGACCCGGTT	CTGAGGCATT	TATATTTATT	GATTTATTTA	ATTCTCATTT	AACCATGAAG	2580
GAGGTACTAT	CACTATCCTT	ATTTTATAGT	TGATAAAGAT	AAAGCCCAGA	GAAATGAATT	2640
AACTCACCCA	AAGTCATGTA	GCTAAGTGAC	AGGGCAAAAA	TTCAAACCAG	TTCCCCAACT	2700
TTACGTGATT	AATACTGTGC	TATACTGCCT	CTCTGATCAT	ATGGCATGGA	ATGCAGACAT	2760
CTGCTCCGTA	AGGCAGAATA	TGGAAGGAGA	TTGGAGGATG	ACACAAAACC	AGCATAATAT	2820
CAGAGGAAAA	GTCCAAACAG	GACCTGAACT	GATAGAAAAA	TTGTTACTCC	TGGTGTAGTC	2880
GCATCGACAT	CTTGATGAAC	TGGTGGCTGA	CACAACATAC	ATTGGCTTGA	TGTGTACATA	2940
TTATTTGTAG	TTGTGTGTGT	ATTTTATAT	ATATATTTGT	AATATTGAAA	TAGTCATAAT	3000
TTACTAAAGG	CCTACCATTT	GCCAGGCATT	TTTACATTTG	TCCCCTCTAA	TCTTTTGATG	3060
AGATGATCAG	ATTGGATTAC	TTGGCCTTGA	AGATGATATA	TCTACATCTA	TATCTATATC	3120
TATATCTATA	TCTATATCTA	TATCTATATC	TATATCTATA	TATGTATATC	AGAAAAGCTG	3180
AAATATGTTT	TGTAAAGTTA	TAAAGATTTT	AGACTTTATA	GAATCTGGGA	TTTGCCAAAT	3240
GTAACCCCTT	TCTCTACATT	AAACCCATGT	TGGAACAAAT	ACATTTATTA	TTTATTTCATC	3300
AAATGTTGCT	GAGTCCTGGC	TATGAACCAG	ACACTGTGAA	AGCCTTTGGG	ATATTTTGCC	3360
CATGCTTGGG	CAAGCTTATA	TAGTTTGCTT	CATAAACTC	TATTTTCAGT	CTTCATAACT	3420
AATACTTCAT	GACTATTGCT	TTTCAGGTAT	TCCTTCATAA	CAAATACTTT	GGCTTTCATA	3480
TATTTGAGTA	AAGTCCCCCT	TGAGGAAGAG	TAGAAGAACT	GCACTTTGTA	AATACTATCC	3540

TGGAATCCAA	ACGGATAGAC	AAGGATGGTG	CTACCTCTTT	CTGGAGAGTA	CGTGAGCAAG	3600
GCCTGTTTTG	TTAACATGTT	CCTTAGGAGA	CAAAACTTAG	GAGAGACACG	CATAGCAGAA	3660
AATGGACAAA	AACTAACAAA	TGAATGGGAA	TTGTACTTGA	TTAGCATTGA	AGACCTTGTT	3720
TATACTATGA	TAAATGTTTG	TATTTGCTGG	AAGTGCTACT	GACGGTAAAC	CCTTTTGTGTT	3780
TAAATGTGTG	CCCTAGTAGC	TTGCAGTATG	ATCTATTTTT	TAAGTACTGT	ACTTAGCTTA	3840
TTTAAAAATT	TTATGTTTAA	AATTGCATAG	TGCTCTTTCA	TTGAAGAAGT	TTTGAGAGAG	3900
AGATAGAATT	AAATTCACTT	ATCTTACCAT	CTAGAGAAAC	CCAATGTTAA	AACTTTGTGTT	3960
TCCATTATTT	CTGTCTTTTA	TTCAACATTT	TTTTTAGAGG	GTGGGAGGAA	TACAGAGGAG	4020
GTACAATGAT	ACACAAATGA	GAGCACTCTC	CATGTATTGT	TTTGTCTGT	TTTTCAGTTA	4080
ACAATATATT	ATGAGCATAT	TTCCATTTCA	TTAAATATTC	TTCCACAAAG	TTATTTTGAT	4140
GGCTGTATAT	CACCCTACTT	TATGAATGTA	CCATATTAAT	TTATTTCTCTG	GTGTGGGTTA	4200
TTTGATTTTA	TAATCTTACC	TTTGAATAAA	TGAAACACCT	GTGAAGCTTT	AGAAAATACT	4260
GGTGCCTGGG	TCTCAACTCC	ACAGATTCTG	ATTTAACTGG	TCTGGGTTAC	AGACTAGGCA	4320
TTGGGAATTC	AAAAAGTTCC	CCCAGTGATT	CTAATGTGTA	GCCAAGATCG	GGAACCCTTG	4380
TAGACAGGGA	TGATAGGAGG	TGAGCCACTC	TTAGCATCCA	TCATTTAGTA	TTAACATCAT	4440
CATCTTGAGT	TGCTAAGTGA	ATGATGCACC	TGACCCACTT	TATAAAGACA	CATGTGCAAA	4500
TAAAATTATT	ATAGGACTTG	GTTTATTAGG	GCTTGTGCTC	TAAGTTTCT	ATGTTAAGCC	4560
ATACATCGCA	TACTAAATAC	TTTAAAATGT	ACCTTATTGA	CATACATATT	AAGTGAAAAG	4620
TGTTTCTGAG	CTAAACAATG	ACAGCATAAT	TATCAAGCAA	TGATAATTTG	AAATGAATTT	4680
ATTATTCTGC	AACTTAGGGA	CAAGTCATCT	CTCTGAATTT	TTTGTACTTT	GAGAGTATTT	4740
GTTATATTTG	CAAGATGAAG	AGTCTGAATT	GGTCAGACAA	TGTCTTGTGT	GCCTGGCATA	4800
TGATAGGCAT	TTAATAGTTT	TAAAGAATTA	ATGTATTTAG	ATGAATTGCA	TACCAAATCT	4860
GCTGTCTTTT	CTTTATGGCT	TCATTAACCT	AATTTGAGAG	AAATTAATTA	TTCTGCAACT	4920
TAGGGACAAG	TCATGTCTTT	GAATATTCTG	TAGTTTGAGG	AGAATATTTG	TTATATTTGC	4980
AAAATAAAAT	AAGTTTGCAA	GTTTTTTTTT	TCTGCCCAA	AGAGCTCTGT	GTCCTTGAAC	5040
ATAAAATACA	AATAACCGCT	ATGCTGTAA	TTATTGGCAA	ATGTCCCAT	TTCAACCTAA	5100
GGAAATACCA	TAAAGTAACA	GATATACCAA	CAAAAGGTTA	CTAGTTAACA	GGCATTGCCT	5160
GAAAAGAGTA	TAAAAGAATT	TCAGCATGAT	TTCCATATT	GTGCTTCCAC	CACTGCCAAT	5220
AACA						5224

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 822 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCATTGCTGT	GAACCTCTGTA	CTTAGGACTA	AACTTTGAGC	AATAACACAC	ATAGATTGAG	60
GATTGTTTGC	TGTTAGCATA	CAAACCTCTG	TTCAAAGCTC	CTCTTTATTG	CTGTCTTG	120
AAAATTTGCT	GTTCTTCATG	GTTTCTCTTT	TCACTGCTAT	CTATTTTCT	CAACCACTCA	180
CATGGCTACA	ATAACTGTCT	GCAAGCTTAT	GATTCCCAA	TATCTATCTC	TAGCCTCAAT	240
CTTGTTCCAG	AAGATAAAAA	GTAGTATTCA	AATGCACATC	AACGTCTCCA	CTGGAGGGC	300
TTAAAGACGT	TTCAACATAC	AAACCGGGGA	GTTTGCCTG	GAATGTTTCC	TAAAATGTGT	360
CCTGTAGCAC	ATAGGGTCCT	CTTGTCCTT	AAAATCTAAT	TACTTTTAGC	CCAGTGCTCA	420
TCCCACCTAT	GGGAGATGA	GAGTGAAAAG	GGAGCTGAT	TAATAATTAC	ACTAAGTCAA	480
TAGGCATAGA	GCCAGGACTG	TTTGGGTAAA	CTGGTCACTT	TATCTTAAAC	TAAATATATC	540
CAAACTGAA	CATGTACTTA	GTTACTAAGT	CTTTGACTTT	ATCTCATTCA	TACCACTCAG	600
CTTTATCCAG	GCCACTTATG	AGCTCTGTGT	CCTTGAACAT	AAAATACAAA	TAACCGCTAT	660

GCTGTTAATT	ATTGGCAAAT	GTCCCATTTT	CAACCTAAGG	AAATACCATA	AAGTAACAGA	720
TATACCAACA	AAAGGTTACT	AGTTAACAGG	CATTGCCTGA	AAAGAGTATA	AAAGAATTTT	780
AGCATGATTT	TCCATATTGT	GCTTCCACCA	CTGCCAATAA	CA		822

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 472 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGCCACCACC	CAGTGAGCCT	TTTTCTAGCC	CCCAGAGCCA	CCTCTGTCAC	CTTCCTGTTG	60
GGCATCATCC	CACCTTCCCA	GAGCCCTGGA	GAGCATGGGG	AGACCCGGGA	CCCTGCTGGG	120
TTTCTCTGTC	ACAAAGGAAA	ATAATCCCCC	TGGTGTGACA	GACCCAAGGA	CAGAACACAG	180
CAGAGGTCAG	CACTGGGGAA	GACAGGTTGT	CCTCCCAGGG	GATGGGGGTC	CATCCACCTT	240
GCCGAAAAGA	TTTGTCTGAG	GAAGTGAAGA	TAGAAGGGAA	AAAAGAGGAG	GGACAAAAGA	300
GGCAGAAATG	AGAGGGGAGG	GGACAGAGGA	CACCTGAATA	AAGACCACAC	CCATGACCCA	360
CGTGATGCTG	AGAAGTACTC	CTGCCCTAGG	AAGAGACTCA	GGGCAGAGGG	AGGAAGGACA	420
GCAGACCAGA	CAGTCACAGC	AGCCTTGACA	AAACGTTTCT	GGAAGTCAAG	CA	472

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 858 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGAGCGGCCC	CTCAGCTTCG	GCGCCCAGCC	CCGCAAGGCT	CCCGGTGACC	ACTAGAGGGC	60
GGGAGGAGCT	CCTGGCCAGT	GGTGGAGAGT	GGCAAGGAAG	GACCCTAGGG	TTCATCGGAG	120
CCCAGGTTTA	CTCCCTTAAG	TGGAAATTTT	TTCCCCCACT	CCTCCTTGGC	TTTCTCCAAG	180
GAGGGAACCC	AGGCTGCTGG	AAAGTCCGGC	TGGGGCGGGG	ACTGTGGGTT	CAGGGGAGAA	240
CGGGGTGTGG	AACGGGACAG	GGAGCGGTTA	GAAGGGTGGG	GCTATTCCGG	GAAGTGGTGG	300
GGGGAGGGAG	CCCAAACTA	GCACCTAGTC	CACTCATTAT	CCAGCCCTCT	TATTTCTCGG	360
CCGCTCTGCT	TCAGTGGACC	CGGGGAGGGC	GGGGAAGTGG	AGTGGGAGAC	CTAGGGGTGG	420
GCTTCCCGAC	CTTGCTGTAC	AGGACCTCGA	CCTAGCTGGC	TTTGTTCCCC	ATCCCCACGT	480
TAGTTGTTGC	CCTGAGGCTA	AAACTAGAGC	CCAGGGGCCC	CAAGTTCCAG	ACTGCCCTC	540
CCCCCTCCCC	CGGAGCCAGG	GAGTGGTTGG	TGAAAGGGGG	AGGCCAGCTG	GAGAACAAAC	600
GGGTAGTCAG	GGGGTTGAGC	GATTAGAGCC	CTTGTACCCT	ACCCAGGAAT	GGTTGGGGAG	660
GAGGAGGAAG	AGGTAGGAGG	TAGGGGAGGG	GGCGGGGTTT	TGTCACCTGT	CACCTGCTCG	720
CTGTGCCTAG	GGCGGGCGGG	CGGGGAGTGG	GGGACCGGT	ATAAAGCGGT	AGGCGCCTGT	780
GCCCCTCCA	CCTCTCAAGC	AGCCAGCGCC	TGCTGAATC	TGTTCTGCCC	CCTCCCCACC	840
CATTTCACCA	CCACCATG					858

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 307 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 2...304
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

G ATG ACC GGC TCA ACC ATC GCG CCC ACA ACG GAC TAT CGC AAC ACC ACT	49
Met Thr Gly Ser Thr Ile Ala Pro Thr Thr Asp Tyr Arg Asn Thr Thr	
1 5 10 15	
GCT ACC GGA CTA ACA TCT GCC CTA AAT TTA CCC CAA GTT CAT GCC TTT	97
Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala Phe	
20 25 30	
GTC AAT GAC TGG GCG AGC TTG GAC ATG TGG TGG TTT TCC ATA GCG CTT	145
Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala Leu	
35 40 45	
ATG TTT GTT TGC CTT ATT ATT ATG TGG CTT ATT TGT TGC CTA AAG CGC	193
Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg	
50 55 60	
AGA CGC GCC AGA CCC CCC ATC TAT AGG CCT ATC ATT GTG CTC AAC CCA	241
Arg Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Ile Val Leu Asn Pro	
65 70 75 80	
CAC AAT GAA AAA ATT CAT AGA TTG GAC GGT CTG AAA CCA TGT TCT CTT	289
His Asn Glu Lys Ile His Arg Leu Asp Gly Leu Lys Pro Cys Ser Leu	
85 90 95	
CTT TTA CAG TAT GAT TAA	307
Leu Leu Gln Tyr Asp	
100	

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 101 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein
- (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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Met Thr Gly Ser Thr Ile Ala Pro Thr Thr Asp Tyr Arg Asn Thr Thr
 1          5          10          15
Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala Phe
      20          25          30
Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala Leu
      35          40          45
Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg
      50          55          60
Arg Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Ile Val Leu Asn Pro
      65          70          75          80
His Asn Glu Lys Ile His Arg Leu Asp Gly Leu Lys Pro Cys Ser Leu
      85          90          95
Leu Leu Gln Tyr Asp
      100

```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GATCACCGGT GTCCACGGCC AGGTGGTGC

29

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GATCACCGGT GCTCACGCCT GTAATCTCAT CAC

33

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATCACCGGT GGTGGGAT GGCATGGCTT TGG

33

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GATCACCGGT AAAGAATCAG TGATCATCCC AAC

33

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GATCCGGCCG TGGTGCTCAC GCCTGTAATC

30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GATCCGGCCG TGTCCACGGC CAGGTGGTGC AG

32

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CTCATTTTCA GTCACCGGTA AGCTTGG

27

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAGCCGCTCC GACACCGGTA CCTC

24

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GATCACCGGT AAGCTTCCAC AAGTGCATTT AGCC

34

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GATCACCGGT CTGTAGGTAT CTGGACCTCA CTG

33

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GATCCGGCCG AAGCTTCCAC AAGTGCATTT AGCC

34

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATCCGGCCG CTGTAGGTAT CTGGACCTCA CTG

33

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTGACCGGTG CATTGCTGTG AACTCTGTA

29

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATAAGTGGCC TGGATAAAGC TGAGTGG

27

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTCACCGGTC TTTGTTATTG GCAGTGCT

28

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATCCAGGCCA CTTATGAGCT CTGTGTCCTT

30

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TATCGGCCCG CATTGCTGTG AACTCT

26

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTACGGCCGC TTTGTTATTG GCAGTG

26

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 786 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CGGTGACCAC	TAGAGGGCGG	GAGGAGCTCC	TGGCCAGTGG	TGGAGAGTGG	CAAGGAAGGA	60
CCCTAGGGTT	CATCGGAGCC	CAGGTTTACT	CCCTTAAGTG	GAAATTTCTT	CCCCCACTCC	120
TCCTTGGCTT	TCTCCAAGGA	GGGAACCCAG	GCTGCTGGAA	AGTCCGGCTG	GGGCGGGGAC	180
TGTGGGTTCA	GGGGAGAACG	GGGTGTGGAA	CGGGACAGGG	AGCGGTTAGA	AGGGTGGGGC	240
TATTCCGGGA	AGTGGTGGGG	GGAGGGAGCC	CAAACTAGC	ACCTAGTCCA	CTCATTATCC	300
AGCCCTCTTA	TTTCTCGGCC	GCTCTGCTTC	AGTGGACCCG	GGGAGGGCGG	GGAAGTGGAG	360
TGGGAGACCT	AGGGGTGGGC	TTCCCCGACCT	TGCTGTACAG	GACCTCGACC	TAGCTGGCTT	420
TGTTCCCCAT	CCCCACGTTA	GTTGTTGCCC	TGAGGCTAAA	ACTAGAGCCC	AGGGGCCCCA	480
AGTTCCAGAC	TGCCCTCCC	CCCTCCCCCG	GAGCCAGGGA	GTGGTTGGTG	AAAGGGGGAG	540
GCCAGCTGGA	GAACAAACGG	GTAGTCAGGG	GGTTGAGCGA	TTAGAGCCCT	TGTACCCTAC	600
CCAGGAATGG	TTGGGGAGGA	GGAGGAAGAG	GTAGGAGGTA	GGGGAGGGGG	CGGGGTTTTG	660
TCACCTGTCA	CCTGCTCGCT	GTGCCTAGGG	CGGGCGGGCG	GGGAGTGGGG	GGACCGGTAT	720
AAAGCGGTAG	GCGCTGTGC	CCGCTCCACC	TCTCAAGCAG	CCAGCGCCTG	CCTGAATCTG	780
TTCTGC						786

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TAATCCGGAC	GGTGACCACT	AGAGGG	26
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(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TATTCCGGAT	CACTTAGGCA	GCGCTG	26
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(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TAACGGCCGC GGTGACCACT AGAG

24

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TATCGGCCGG CAGAACAGAT TCAG

24

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ATTACCGGTA GCCACCACCC AGTGAG

26

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TAGACCGGTG CTTGAGTTCC AGGAAC

26

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TAACGGCCGA GCCACCACC A

21

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TATCGGCCGG CTTGAGTTCC AGG

23

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GCCTTAATTA AAAGCAAACC TCACCTCCG

29

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GTGGAACAAA AGGTGATTAA AAAATCCCAG

30

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CACCTTTTGT TCCACCGCTC TGCTTATTAC

30

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GGCTTAATTA ACTGTGAAAG GTGGGAGC

28

CLAIMS

What is claimed is:

- 5 1. An adenovirus vector comprising a first adenovirus gene under transcriptional control of a first heterologous transcriptional regulatory element (TRE) and at least a second gene under transcriptional control of a second heterologous TRE, wherein the first heterologous TREs is cell-specific, the first heterologous TRE is different from the second heterologous TRE, and the heterologous TREs are functional in the same cell.
- 10 2. The adenovirus vector of claim 1, wherein the first adenovirus gene is essential for adenovirus replication.
3. The adenovirus vector of claim 2, wherein the gene essential for
15 replication is an adenovirus early gene.
4. The adenovirus vector of claim 3, wherein the gene essential for replication is the adenovirus E1A gene.
- 20 5. The adenovirus vector of claim 3, wherein the gene essential for replication is the adenovirus E1B gene.
6. The adenovirus vector of claim 3, wherein the gene essential for
25 replication is the adenovirus E4 gene.
7. The adenovirus vector of claim 2, wherein the gene essential for replication is an adenovirus late gene.

8. The adenovirus vector of claim 1, wherein the first and second genes are essential for adenovirus replication.

9. The adenovirus vector of claim 8, wherein the first and second genes are adenovirus early genes.

10. The adenovirus vector of claim 8, wherein one gene essential for replication is an adenovirus early gene and one gene essential for replication is an adenovirus late gene.

11. The adenovirus vector of claim 1, wherein the second gene is a transgene.

12. The adenovirus vector of claim 11, wherein said transgene is a cytotoxic gene.

13. The adenovirus vector of claim 1, wherein the adenoviral gene is the adenovirus death protein gene.

14. The adenovirus vector of claim 2, wherein the second gene is a transgene.

15. The adenovirus vector of claim 14, wherein said transgene is a cytotoxic gene.

16. The adenovirus vector of claim 2, wherein the second gene is the adenovirus death protein gene.

17. The adenovirus vector of claim 1, wherein the first and second heterologous TREs are cell specific and functional in the same cell.

18. The adenovirus vector of claim 1, wherein a third gene is under transcriptional control of a third heterologous TRE and the third heterologous TRE is different from the first and the second heterologous TREs.

5

19. The adenovirus vector of claim 1, wherein an additional gene is under transcriptional control of the second heterologous TRE.

20. The adenovirus vector of claim 1, wherein the first heterologous TRE is prostate cell-specific.

10

21. The adenovirus vector of claim 20, wherein the first heterologous TRE is a *PSE*-TRE.

22. The adenovirus vector of claim 20, wherein the first heterologous TRE is a *PB*-TRE.

15

23. The adenovirus vector of claim 20, wherein the first heterologous TRE is an *hKLK2*-TRE.

20

24. The adenovirus vector of claim 1, wherein the first heterologous TRE is liver cell-specific.

25. The adenovirus vector of claim 24, wherein the first heterologous TRE is a *AFP*-TRE.

25

26. The adenovirus vector of claim 1, wherein the first heterologous TRE is breast cancer cell-specific.

27. The adenovirus vector of claim 26, wherein the first heterologous TRE is a *MUC1*-TRE.

5 28. The adenovirus vector of claim 26, wherein the first heterologous TRE is a *CEA*-TRE.

29. The adenovirus vector of claim 1, wherein the first heterologous TRE is colon cancer cell-specific.

10

30. The adenovirus vector of claim 29, wherein the first heterologous TRE is a *CEA*-TRE.

31. A host cell comprising the adenovirus vector of claim 1.

15

32. A composition comprising the adenovirus vector of claim 1.

33. A method for using the adenovirus vector of claim 1 comprising introducing said vector into a cell.

20

34. A method according to claim 33, wherein the cell is a mammalian cell.

35. A method according to claim 34, wherein the mammalian cell is a prostate cell.

25

36. A method according to claim 34, wherein the mammalian cell is a liver cell.

37. A method according to claim 34, wherein the mammalian cell is a breast cancer cell.

38. A method according to claim 34, wherein the mammalian cell is a colon cancer cell.

39. A method for conferring selective cytotoxicity on a cell which allows a heterologous TRE to function, comprising contacting said cell with an adenovirus vector of claim 1, wherein the adenovirus vector enters the cell.

40. A method according to claim 39, wherein cell is a mammalian cell.

41. A method according to claim 40, wherein the mammalian cell is a prostate cell.

42. A method according to claim 40, wherein the mammalian cell is a liver cell.

43. A method according to claim 40, wherein the mammalian cell is a breast cancer cell.

44. A method according to claim 40, wherein the mammalian cell is a colon cancer cell.

45. A method for propagating an adenovirus vector of claim 1, said method comprising combining an adenovirus vector of claim 1 with cells which allow function of the first heterologous TRE, whereby said adenovirus is propagated.

46. A method according to claim 45, wherein the cells are mammalian cells.

47. A method for suppressing tumor growth comprising contacting a target cell with an adenovirus vector according to claim 1 such that the adenovirus vector is introduced into the target cell.

48. A method according to claim 47, wherein the target cell is a mammalian cell.

49. A method according to claim 48, wherein the mammalian cell is a prostate cell.

50. A method according to claim 48, wherein the mammalian cell is a liver cell.

51. A method according to claim 48, wherein the mammalian cell is a breast cancer cell.

52. A method according to claim 48, wherein the mammalian cell is a colon cancer cell.

53. A method for modifying the genotype of a target cell comprising contacting the target cell with the adenovirus vector according claim 1 such that the adenovirus vector is introduced into the target cell.

54. An adenovirus comprising an adenoviral vector of claim 1, wherein the adenovirus is complexed with a masking agent.

55. The adenovirus of claim 54 wherein the masking agent is polyethyleneglycol (PEG).

56. The adenovirus of claim 55, wherein the PEG is of a molecular weight between about 2500 to about 30,000.

57. The adenovirus of claim 56, wherein the PEG is of a molecular weight between about 3000 to about 20,000.

5 58. The adenovirus of claim 57, wherein the PEG is of a molecular weight between about 5000 to about 10,000.

59. The adenovirus of claim 55, wherein the PEG is covalently attached to the adenovirus.

10 60. The adenovirus of claim 55, wherein the PEG is non-covalently attached to the adenovirus.

61. The adenovirus of claim 59, wherein the PEG is covalently attached by using a N-hydroxysuccinimidyl (NHS) active ester.

15 62. The adenovirus of claim 61, wherein the N-hydroxysuccinimidyl (NHS) active ester is selected from the group consisting of succinimidyl succinate, succinimidyl succinamide and succinimidyl propionate.

63. The adenovirus of claim 62, wherein the N-hydroxysuccinimidyl (NHS) active ester is succinimidyl succinate.

20 64. A method of making a masked adenovirus, comprising covalently attaching a masking agent to an adenovirus, wherein the masking agent has a molecular weight between about 2500 and about 20,000, thereby producing a masked adenovirus.

65. The method of claim 64, wherein the masking agent is polyethyleneglycol (PEG).

66. An adenovirus complexed with a masking agent.

67. The adenovirus of claim 66 wherein the masking agent is polyethyleneglycol (PEG).

68. The adenovirus of claim 67, wherein the PEG is of a molecular weight between about 2500 to about 30,000.

5 69. The adenovirus of claim 68, wherein the PEG is of a molecular weight between about 3000 to about 20,000.

70. The adenovirus of claim 69, wherein the PEG is of a molecular weight between about 5000 to about 10,000.

10 71. The adenovirus of claim 67, wherein the PEG is covalently attached to the adenovirus.

72. The adenovirus of claim 67, wherein the PEG is non-covalently attached to the adenovirus.

73. The adenovirus of claim 71, wherein the PEG is covalently attached by using a N-hydroxysuccinimidyl (NHS) active ester.

15 74. The adenovirus of claim 73, wherein the N-hydroxysuccinimidyl (NHS) active ester is selected from the group consisting of succinimidyl succinate, succinimidyl succinamide and succinimidyl propionate.

20 75. The adenovirus of claim 74, wherein the N-hydroxysuccinimidyl (NHS) active ester is succinimidyl succinate.

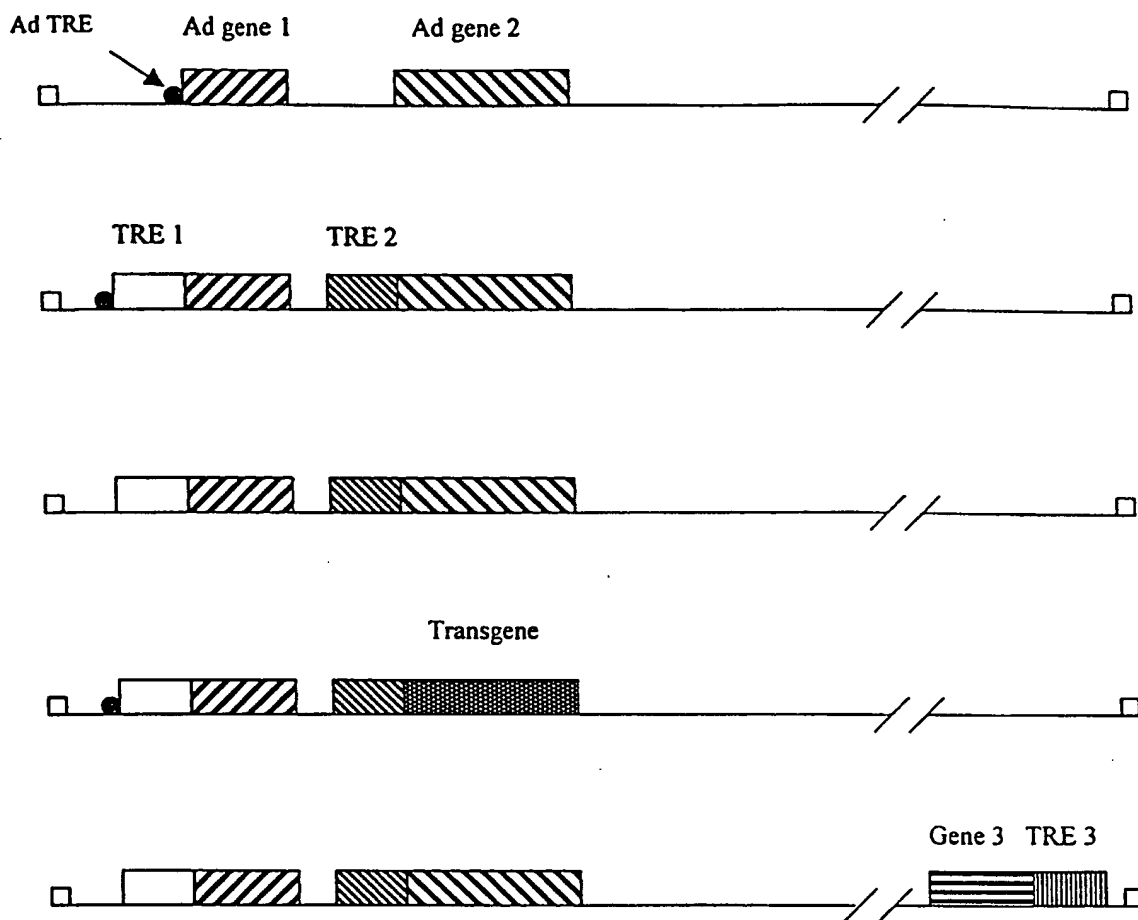


FIGURE 1

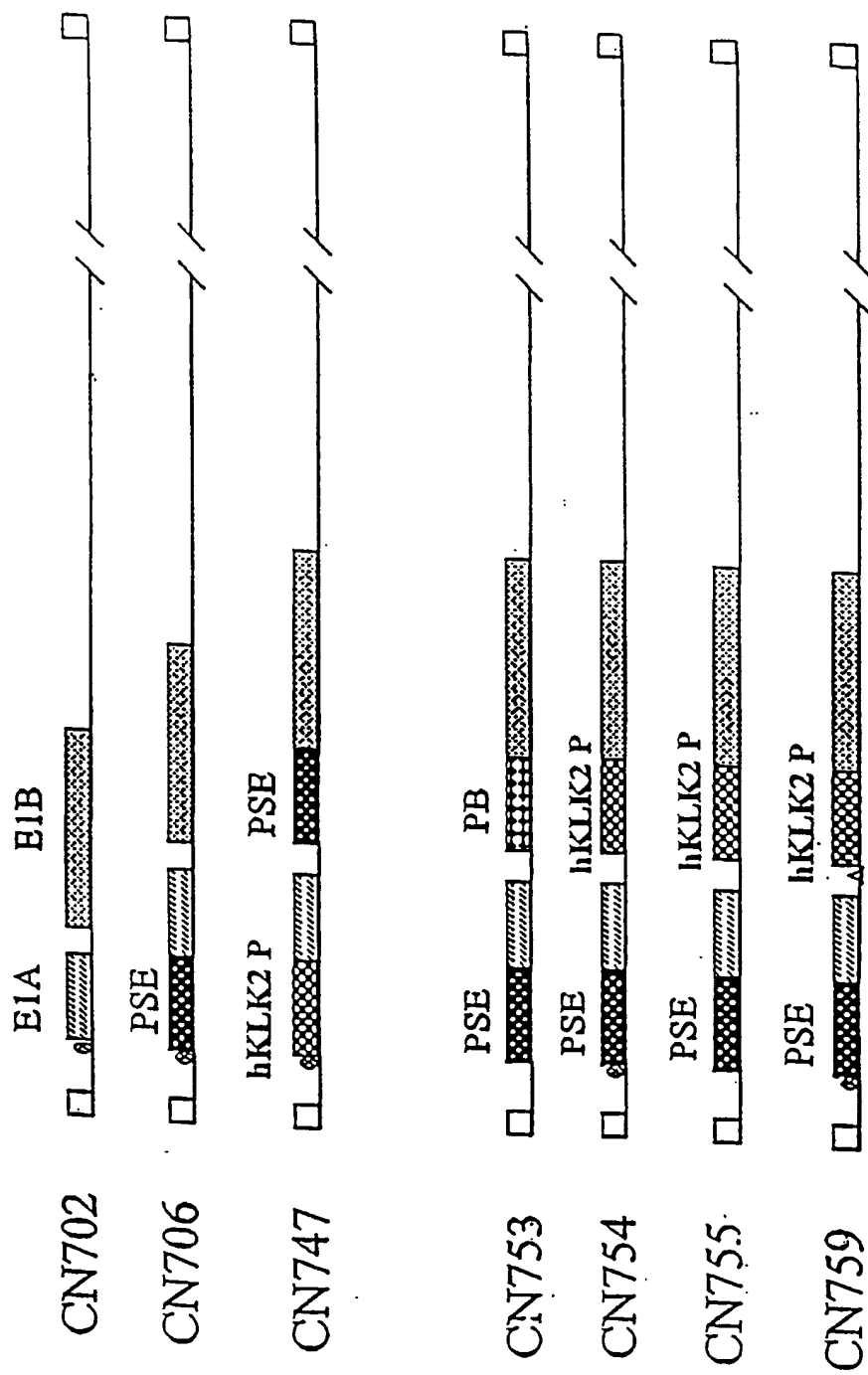


FIGURE 2A

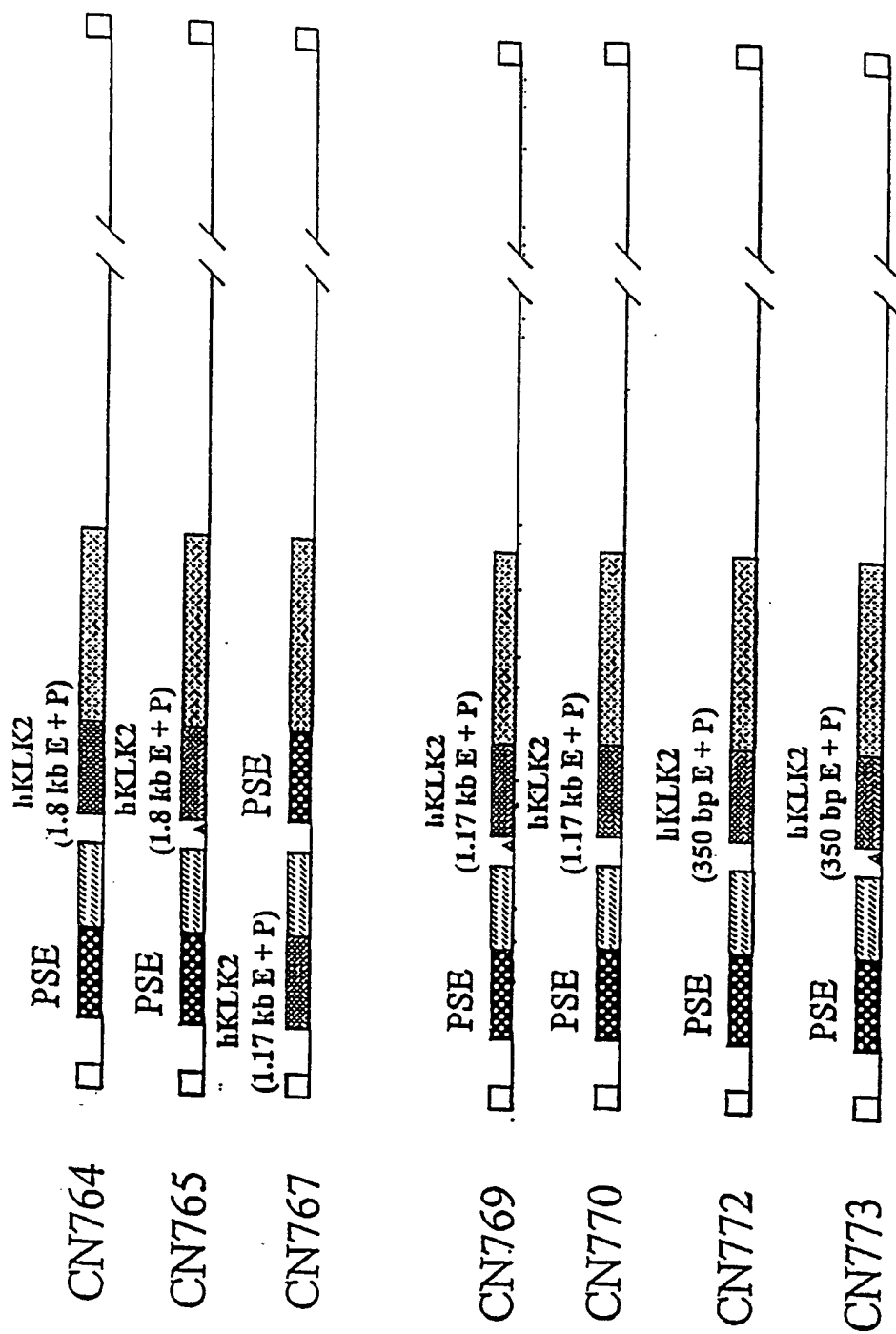


FIGURE 2B

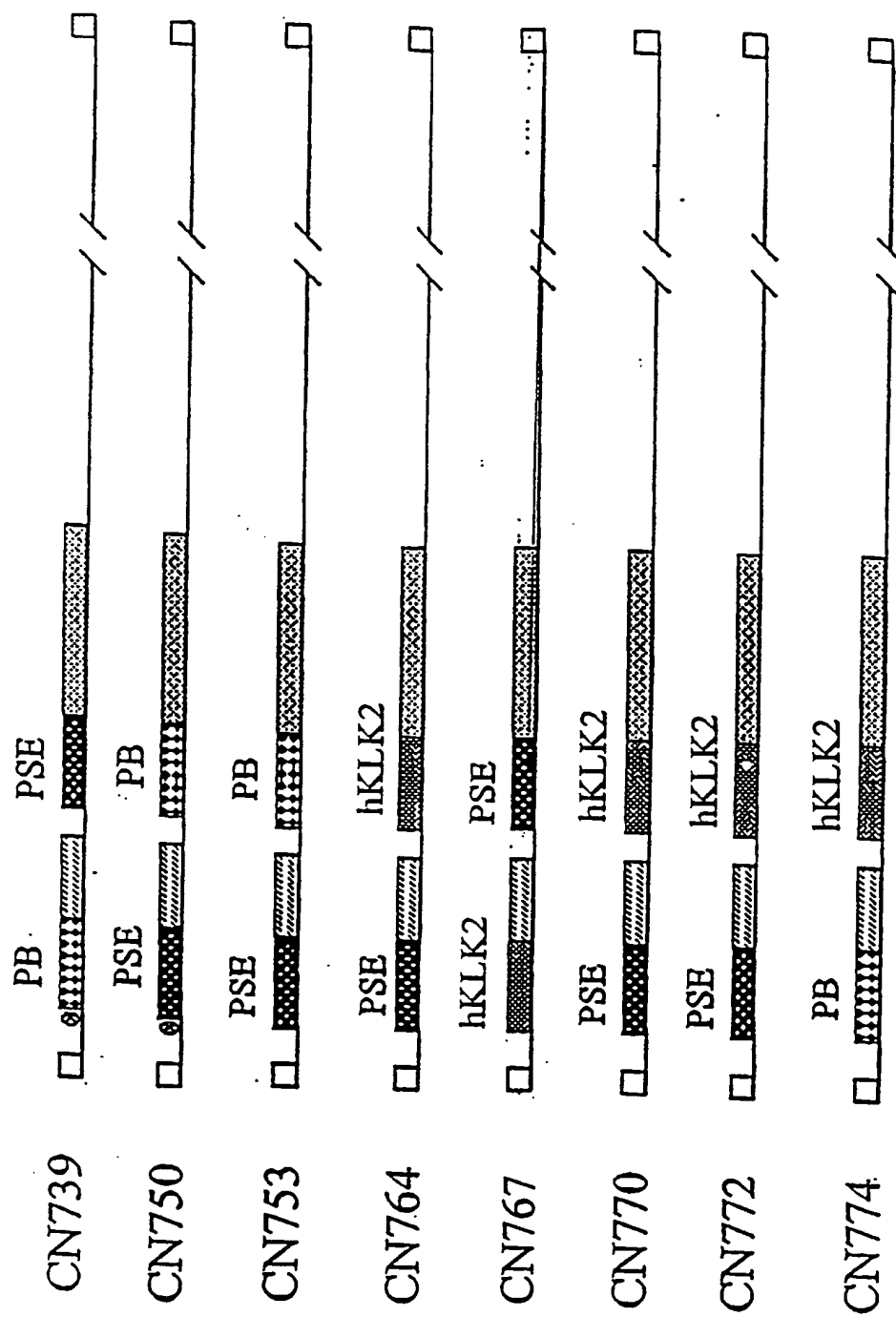


FIGURE 2C

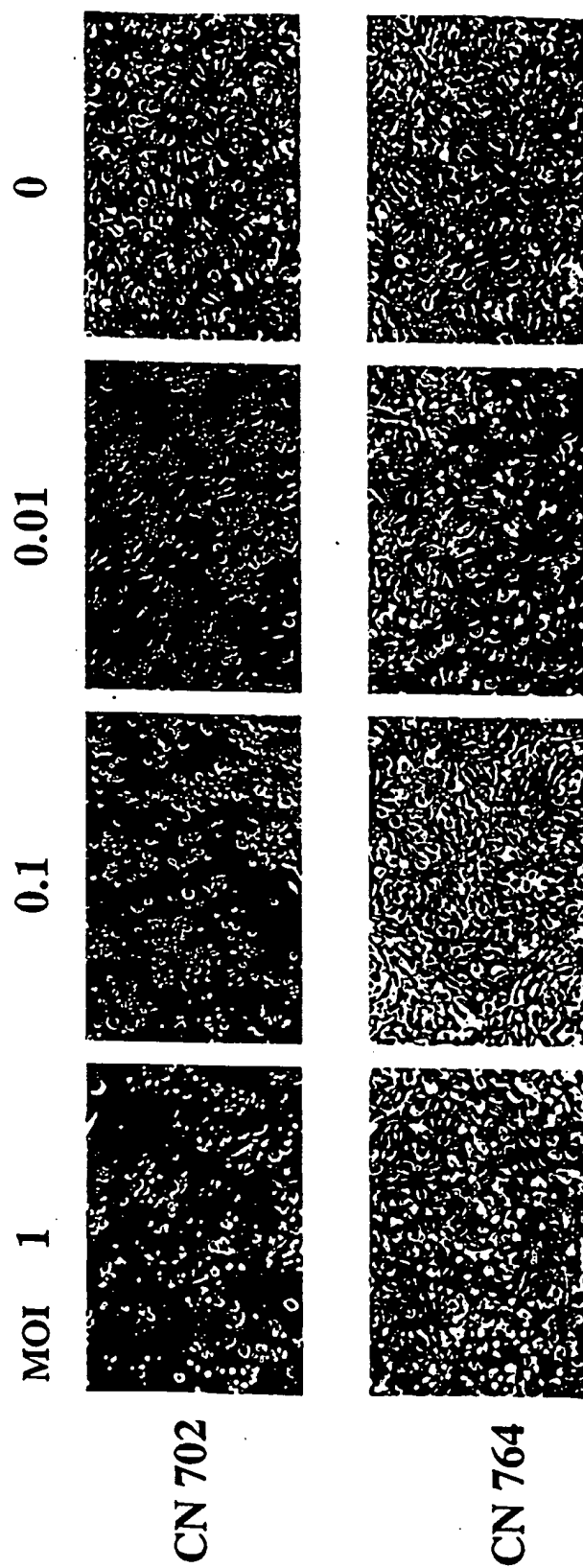


FIGURE 3

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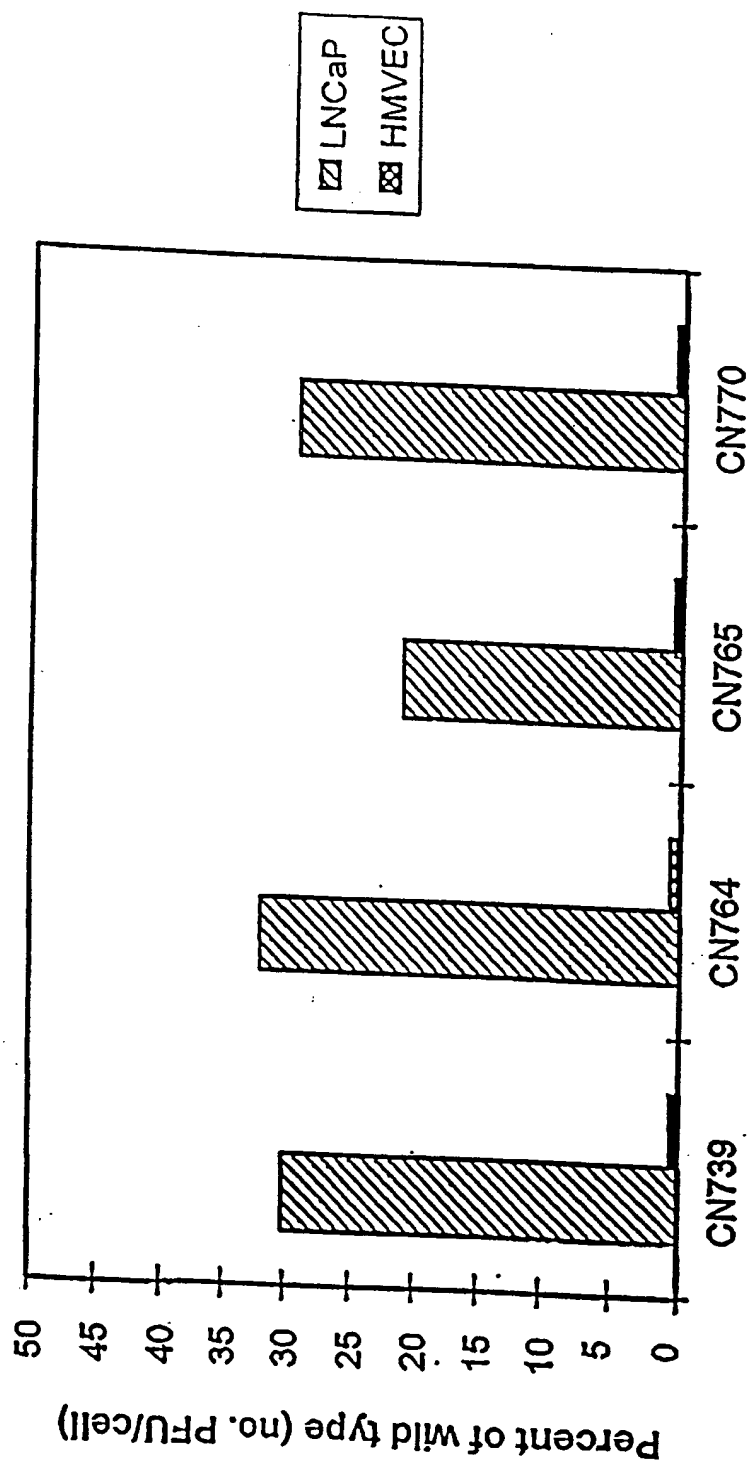


FIGURE 4

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Sheet 1

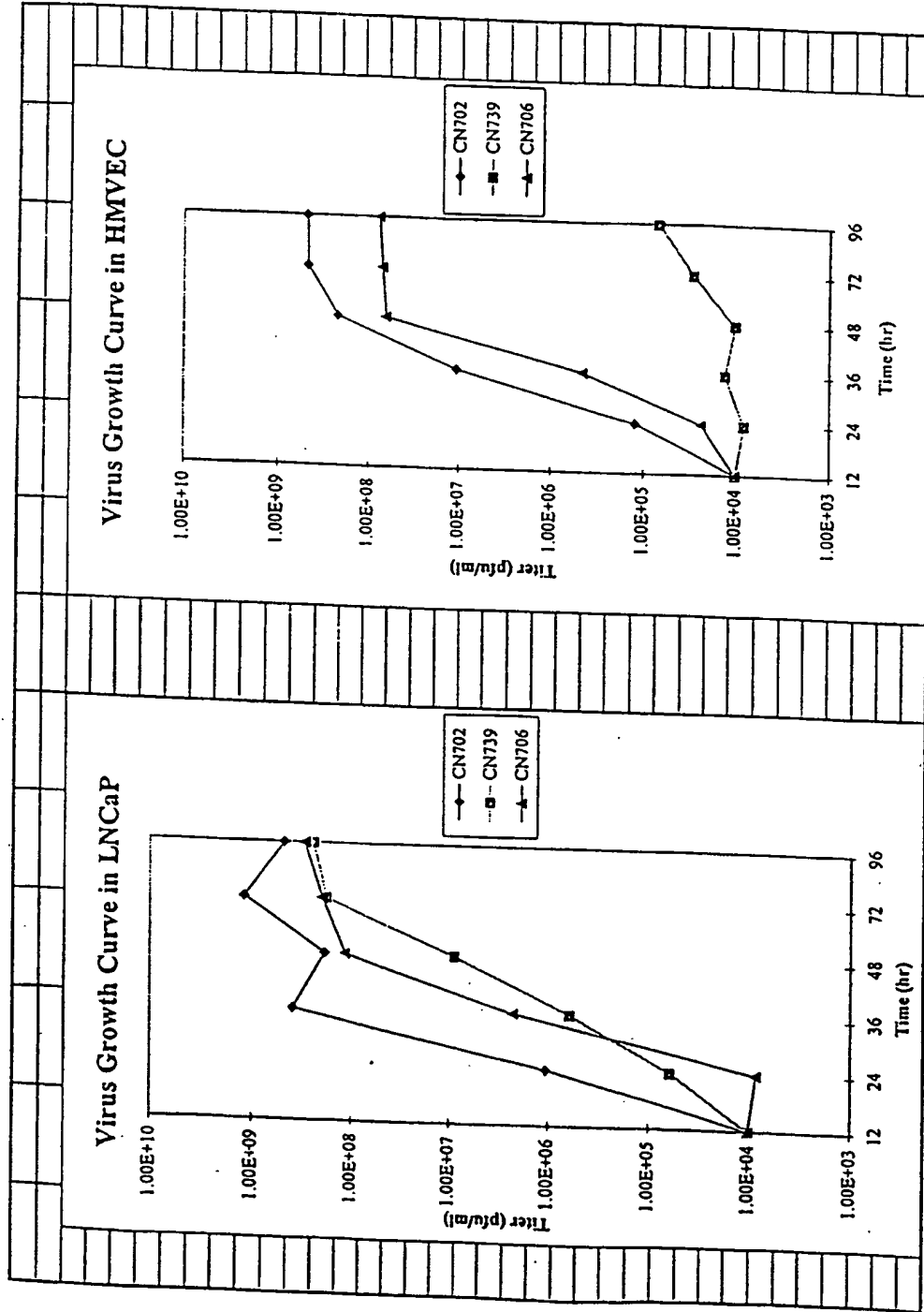


FIGURE 5

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**Efficacy of CN739 in LNCaP Nude Mice Xenograft
(one dose: 2.5×10^8 per mm³)**

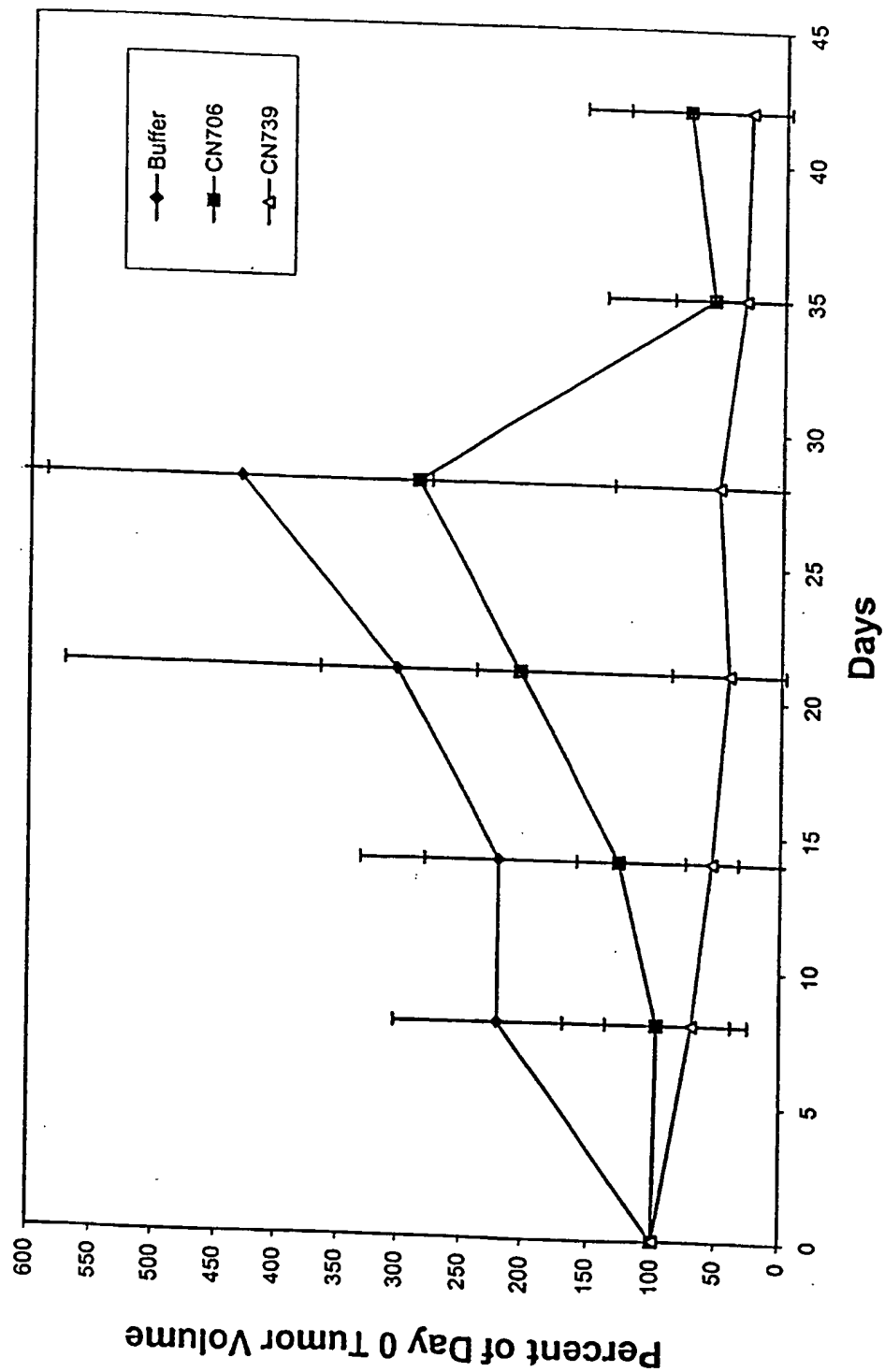


FIGURE 6

PSA Levels of LNCaP Tumor Bearing Nude Mice

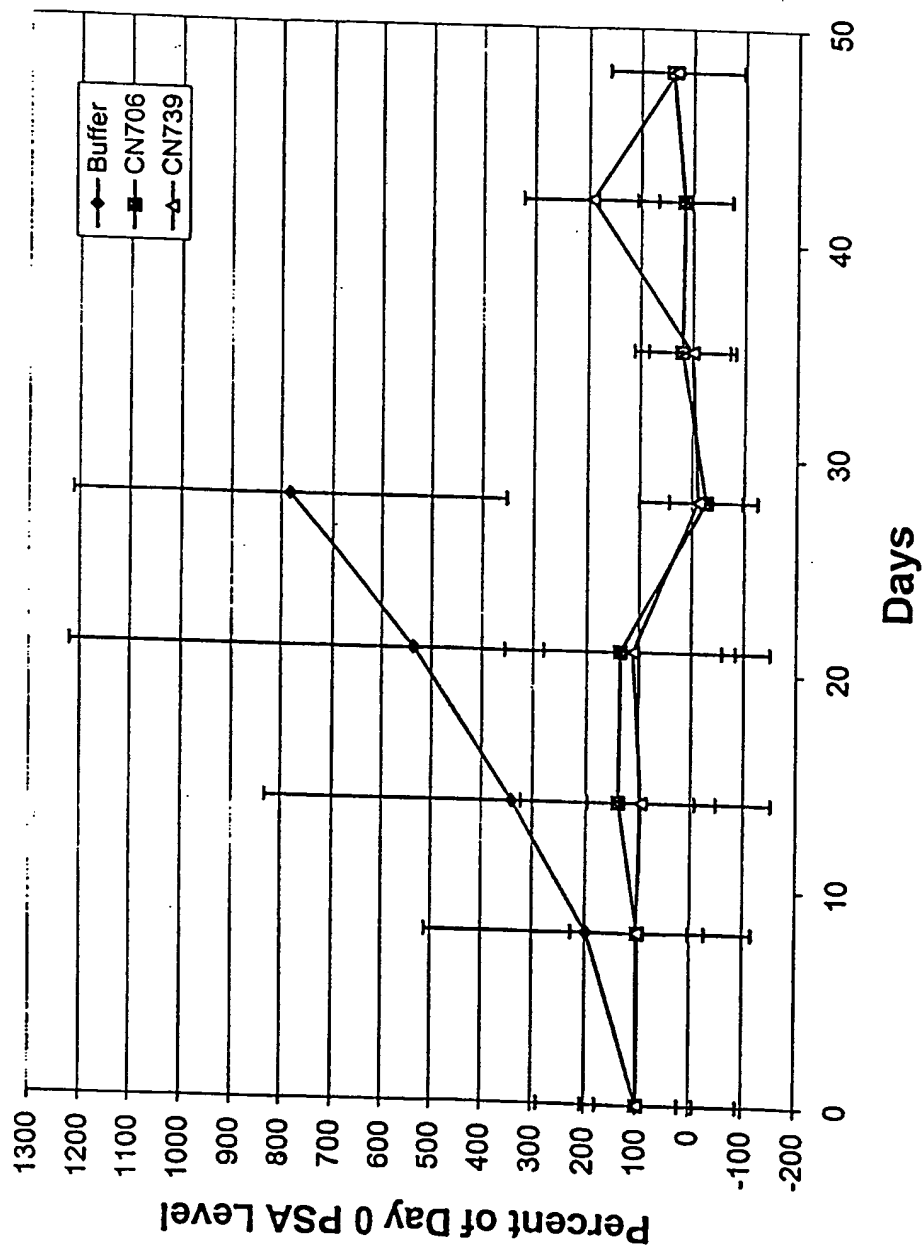


FIGURE 7

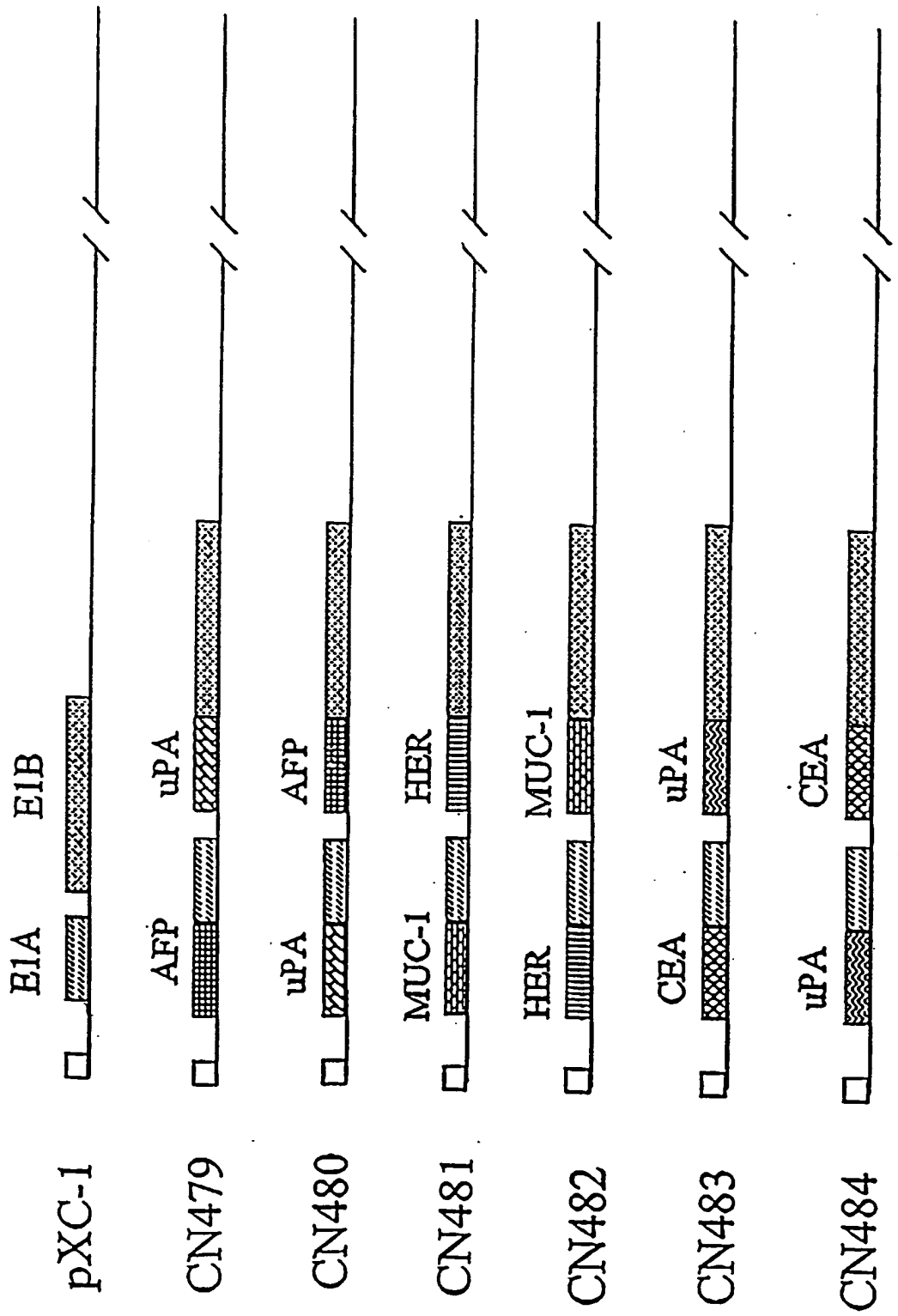


FIGURE 8A.

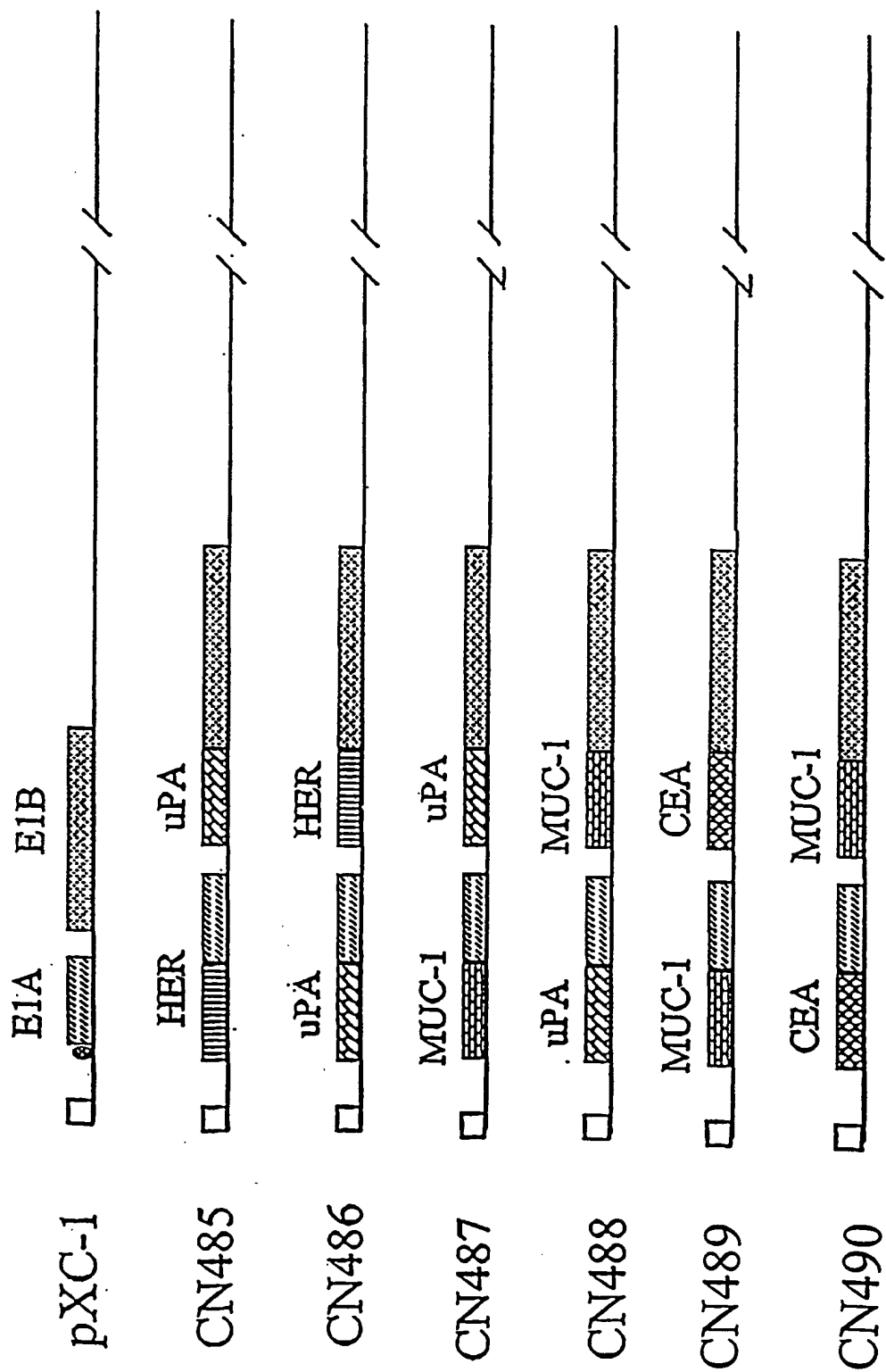


FIGURE 8B

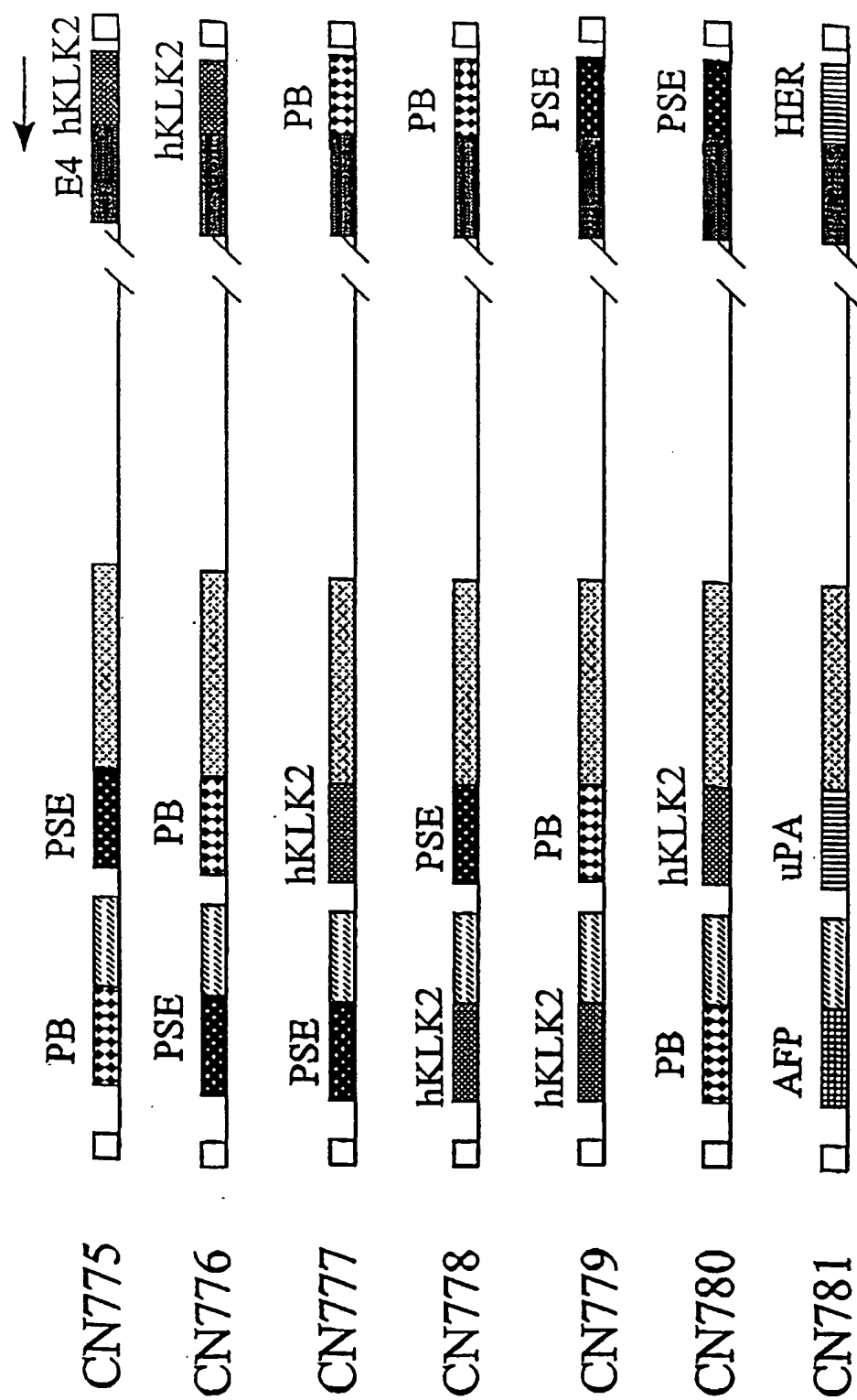


FIGURE 9

Cytotoxicity of Ad Viral Vectors in LNCaP cells

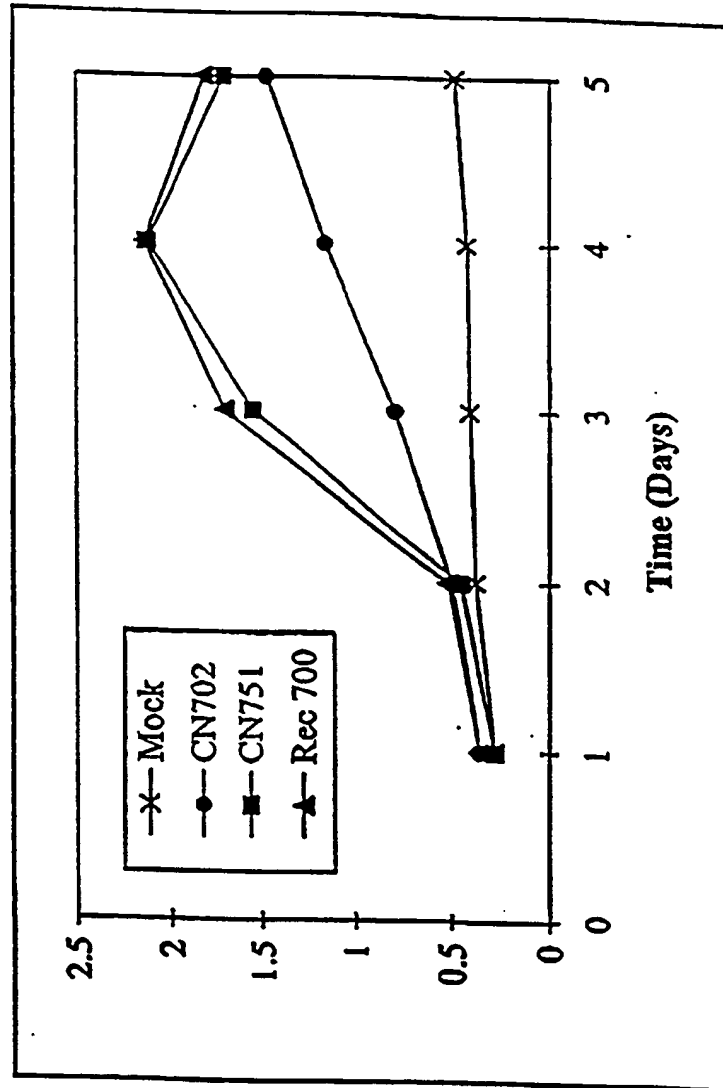


FIGURE 10

Extracellular Virus Yield in Ad Infected A549 Cells

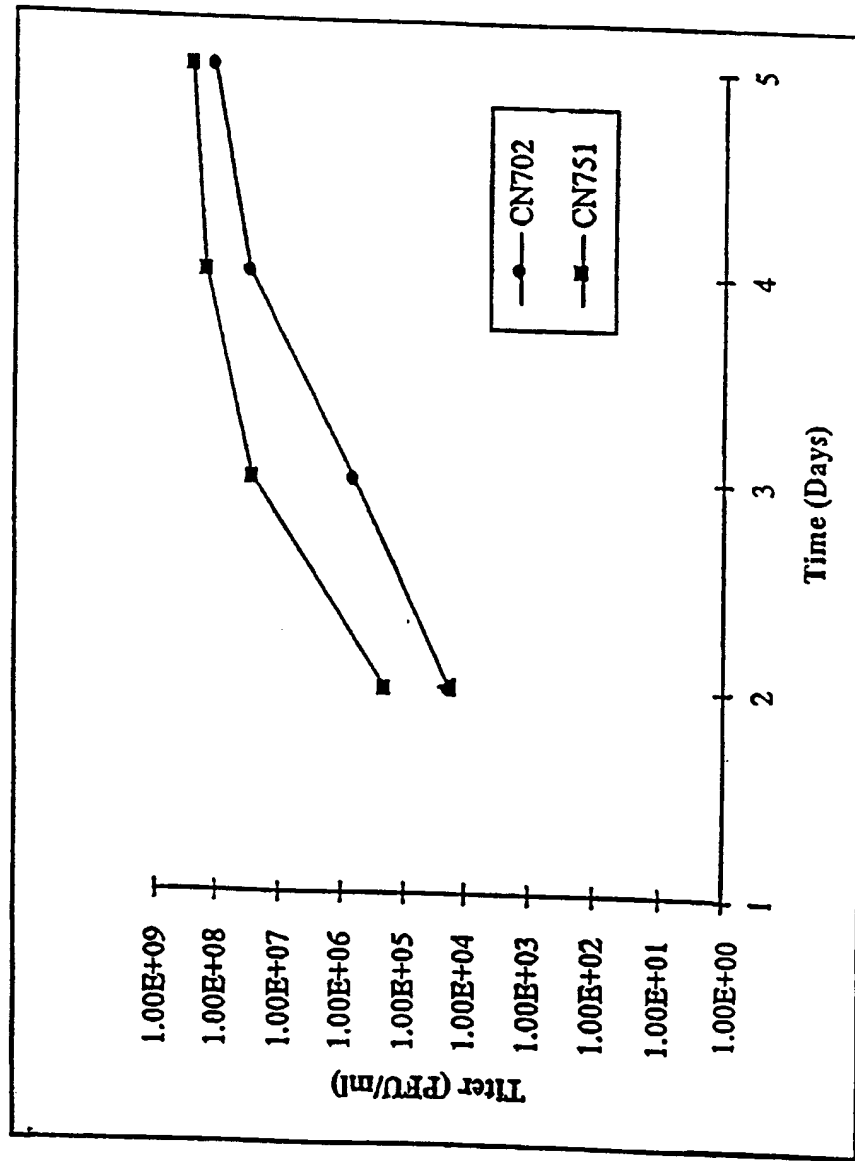
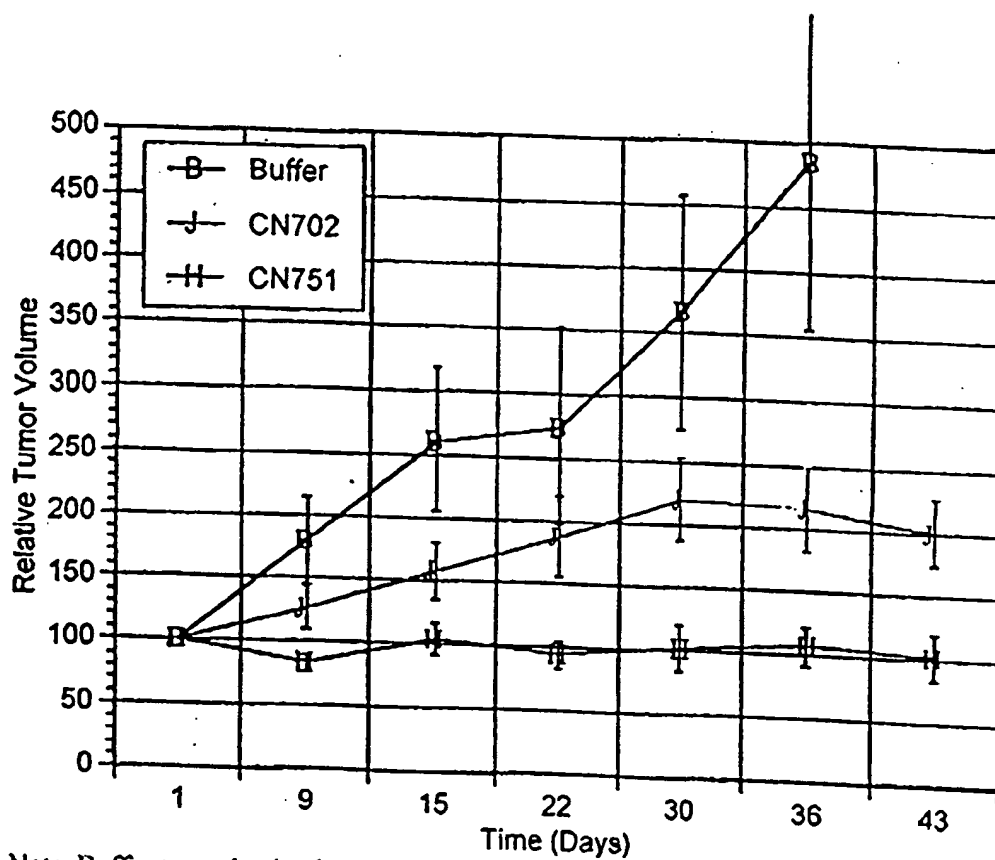


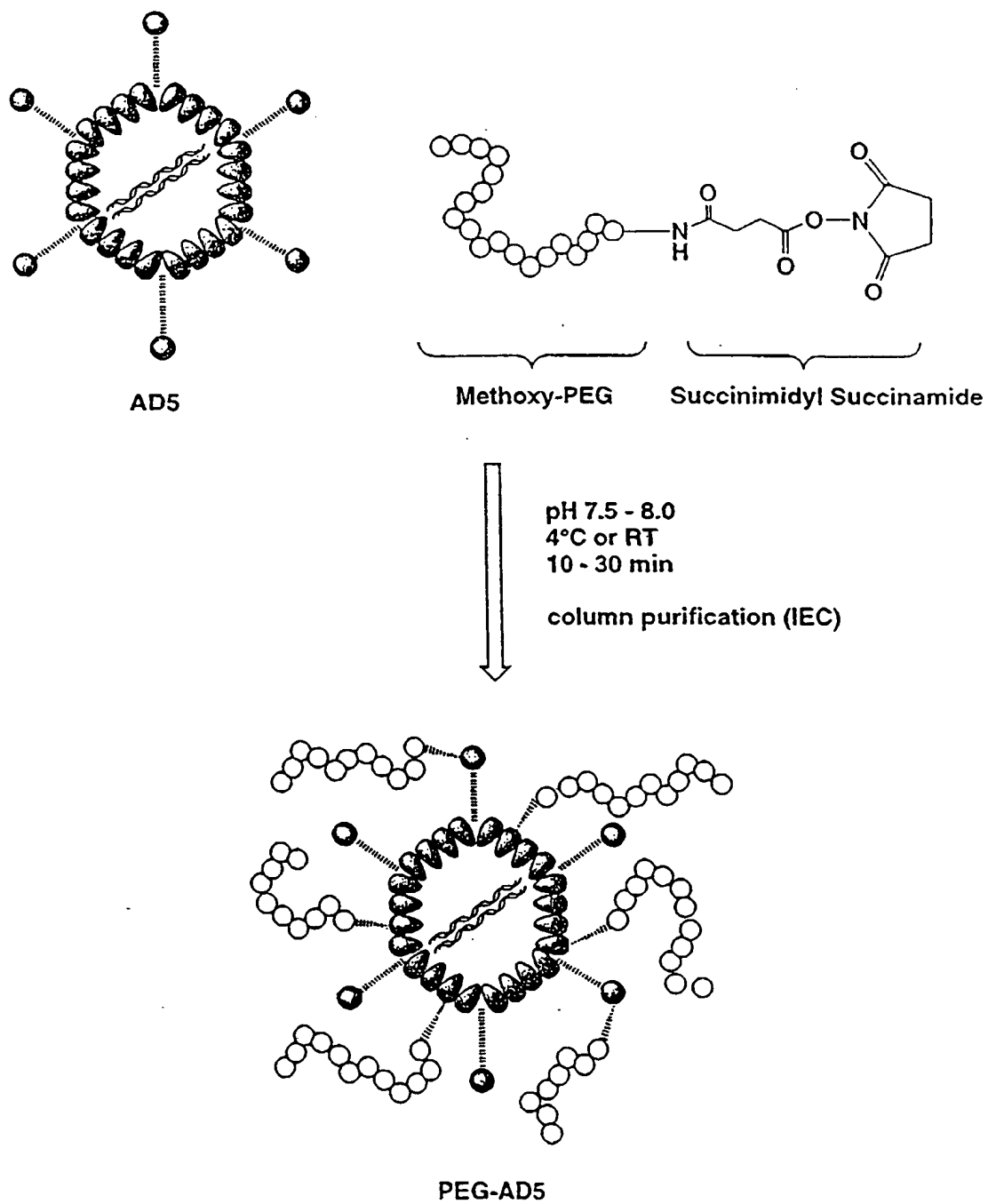
FIGURE 11



Note: Buffer treated animals were sacrificed after four weeks because of excessive tumor burden

FIGURE 12

FIGURE 13



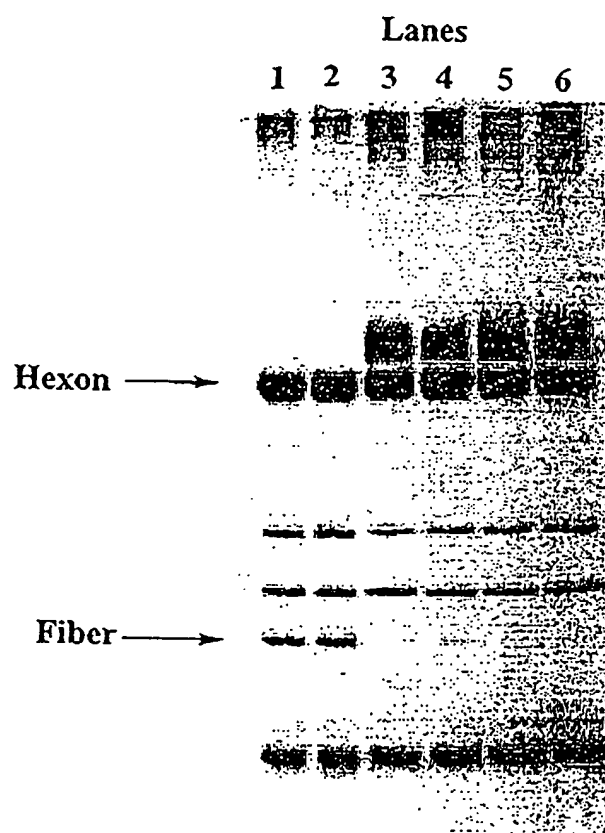


FIGURE 14

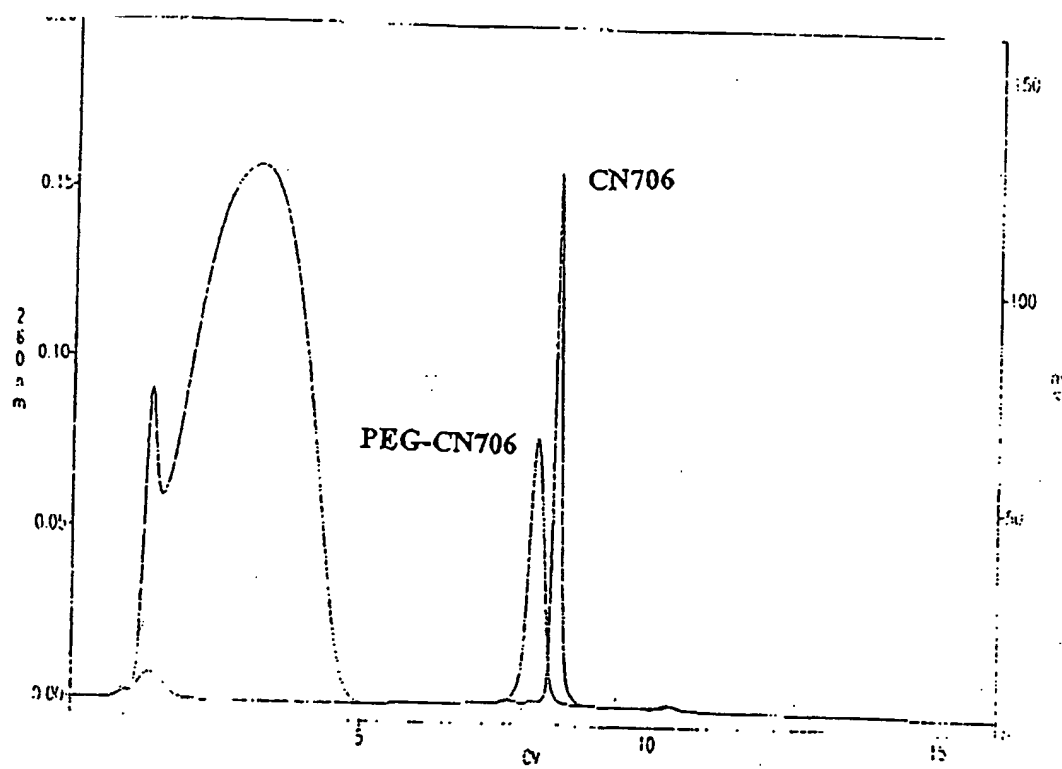


FIGURE 15



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/86, 5/10, A61K 48/00, 47/48, C12N 11/08	A3	(11) International Publication Number: WO 98/39464 (43) International Publication Date: 11 September 1998 (11.09.98)
(21) International Application Number: PCT/US98/04080 (22) International Filing Date: 3 March 1998 (03.03.98) (30) Priority Data: 60/039,762 3 March 1997 (03.03.97) US 60/039,763 3 March 1997 (03.03.97) US 60/054,523 4 August 1997 (04.08.97) US Not furnished 2 March 1998 (02.03.98) US (71) Applicant (for all designated States except US): CALYDON, INC. [US/US]; 1324 Chesapeake Terrace, Sunnyvale, CA 94089 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HENDERSON, Daniel, R. [US/US]; 955 Matadero Avenue, Palo Alto, CA 94306 (US). YU, De-Chao [CN/US]; 1046 Eagle Lane, Foster City, CA 94404 (US). LAMPARSKI, Henry, G. [CA/US]; 422 South El Dorado, San Mateo, CA 94402 (US). (74) Agents: POLIZZI, Catherine, M. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 7 January 1999 (07.01.99)
(54) Title: ADENOVIRUS VECTORS CONTAINING HETEROLOGOUS TRANSCRIPTION REGULATORY ELEMENTS AND METHODS OF USING SAME (57) Abstract <p>Replication-competent adenovirus vectors specific for target cells and methods of use of such viruses are provided. These adenoviruses comprise a first adenoviral gene under control of a cell specific heterologous (i.e., non-adenoviral) transcriptional regulatory element (TRE) and at least a second gene under control of a second heterologous TRE, where the heterologous TREs are different from each other in polynucleotide sequence but functional in the same cell. The adenoviral gene can be, for example, a gene required for adenoviral replication. The second gene can be, for example, a second adenoviral gene or a transgene, such as a gene which can contribute to cytotoxicity in the target cell. Adenoviral replication can be restricted to target cells in which the heterologous TREs are functional and thus, the adenovirus vectors can provide selective cytotoxicity to the target cells, particularly neoplastic cells.</p>		

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INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 98/04080

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C12N5/10 A61K48/00 A61K47/48 C12N11/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 17053 A (GENETIC THERAPY INC ;HALLENBECK PAUL L (US); CHANG YUNG NIEN (US);) 6 June 1996 cited in the application	1-12,14, 15, 17-19, 24-34, 36-40, 42-48, 50-53
Y	see the whole document, especially page 16, lines 12-23 --- -/--	20,21, 35,41,49

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

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P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

8 document member of the same patent family

Date of the actual completion of the international search

10 July 1998

Date of mailing of the international search report

26.10.98

Name and mailing address of the ISA

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Authorized officer

Mandl, B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/04080

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 01358 A (CALYDON) 16 January 1997	1-12,14, 15, 17-21, 31-35, 39-41, 45-49,53
Y	see the whole document	13,16, 22-30, 36-38, 42-44, 50-52
Y	--- WO 96 34969 A (CANJI INC) 7 November 1996	20,21, 28-30, 35,38, 41,44, 49,52
Y	see the whole document --- TOLLEFSON A. E. ET AL.: "The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells." JOURNAL OF VIROLOGY, vol. 70, no. 4, April 1996, pages 2296-2306, XP002071004 see the whole document, especially page 2305, left column, last two lines and right column, first paragraph ---	13,16
Y	--- WO 94 03594 A (UNIV MANITOBA ;MATUSIK ROBERT J (CA)) 17 February 1994 see the whole document ---	22
Y	--- MURTHA P. ET AL.: "Androgen induction of a human prostate-specific kallikrein, hK2: Characterization of an androgen response element in the 5' promoter region of the gene." BIOCHEMISTRY, vol. 32, 1993, pages 6459-6464, XP002071006 cited in the application see the whole document ---	23
Y	--- ARBUTHNOT P. B. ET AL.: "In vitro and in vivo hepatoma cell-specific expression of a gene transferred with an adenoviral vector." HUMAN GENE THERAPY, vol. 7, no. 13, August 1996, pages 1503-1514, XP002071007 see the whole document ---	24,25, 36,42,50
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/04080

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>MANOME Y. ET AL.: "Enhancer sequences of the DF3 gene regulate expression of the herpes simplex virus thymidine kinase gene and confer sensitivity of human breast cancer cells to ganciclovir." CANCER RESEARCH, vol. 54, 1994, pages 5408-5413, XP002071008 see the whole document ---</p>	<p>26,27, 37,43,51</p>
A	<p>WO 95 14100 A (WELLCOME FOUND ;RICHARDS CYNTHIA ANN (US); HUBER BRIAN (US)) 26 May 1995 cited in the application see the whole document ---</p>	<p>28-30, 38,44,52</p>
A	<p>CHEN L. ET AL.: "Breast cancer selective gene expression and therapy mediated by recombinant adenoviruses containing the DF3/ MUC1 promoter." JOURNAL OF CLINICAL INVESTIGATION, vol. 96, no. 6, December 1995, pages 2775-2782, XP002071017 see the whole document -----</p>	<p>26,27, 37,43,51</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/04080

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 33-44 and 47-53, as far as they refer to an in vivo application, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see further information sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-63

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-63

An adenovirus vector comprising an adenovirus gene under transcriptional control of a first tissue-specific heterologous transcriptional regulatory element (TRE) and at least a second gene under transcriptional control of a different second heterologous TRE; a composition comprising said adenovirus vector; a host cell transformed with said adenovirus vector; a method of using said vector comprising introducing said vector into a cell; a method for conferring selective cytotoxicity on a target cell; a method for propagating said adenovirus vector; a method for suppressing tumor growth; and a method for modifying the genotype of a target cell.

2. Claims: 64-75

A method of making a masked adenovirus; and adenovirus masked with a masking agent.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/04080

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